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Observations on the Influence of Force Breeding in Mice With and Without the Mammary-Tumor Agent [1, 2]

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SUMMARY

Females of cancerous strains Z (C3H) and D2 (DBA/2), and agent-free Zb and reciprocal (Ax × Zb)F_1 females were subjected to force breeding and compared with normal breeding females allowed to nurse their young. There was no significant increase in incidence and no correlation between the time of development of mammary cancer and the number of litters in the force breeders, as compared with normal breeders. In this respect, females of subline Z (C3H) differed from animals of either sublines C3H/An or C3H/He, which confirmed the observations made previously by many workers that animals of one line should never serve as controls for experimental data obtained for mice of another subline of the same inbred strain. Comparable experimental observations have been secured by the subjection of mice of the same subline to the functional breeding test and excessive hormonal stimulation. These data would not support the theory suggested by Bagg in 1939 that prevention of nursing produced stagnation of the mammary ducts and became a major factor in the development and/or acceleration of mammary cancer in mice. Earlier it was shown that there was little milk production in the mammary glands of females that had not nursed their young and consequently little milk stagnation. A general review of the subject is included.—J. Nat. Cancer Inst. 26: 1043–1057, 1961.

SINCE THE early investigations of Bagg (*1*, *2*), Fekete and Green (*3*), and others upon the possible influence of stagnation in the genesis of mammary cancer in mice, many workers have subjected females to force breeding and nonsuckling, or Bagg's so-called "functional test" (*1*, *2*, *4–9*). Such females are not permitted to nurse their young and are returned to the breeding pen soon after their young are born, or the females remain with the male and the young are removed.

Other pertinent studies in this field will be found in recent reports by Boot and Mühlbock (*10*), Bittner (*11*), Deringer (*12*), and Dmochowski (*13*). In addition to force breeding, Fekete (*14*) studied other breeding

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tests in attempts to produce functional activity of the mammary glands associated with the development of mammary cancer. Most investigators have been interested in force breeding to increase the incidence and to accelerate the time of development of mammary tumors in animals of strains having low incidences.

In his analysis of females of the cancerous DBA stock, Murray (*15, 16*) was unable to find any correlation between the genesis of this type of cancer and whether the females nursed all or none of their litters. It should be pointed out that these females may not be directly comparable to force breeders, since they were permitted to nurse their young for various periods; also the females that lost their litters at different ages were not necessarily returned to the breeding pens as soon as their young died. Regardless of these differences, considerable stagnation of milk in the mammary glands would be expected to occur and, according to Bagg (*5*), was the primary cause of cancer in mice exposed to his "functional test."

Equivocal results were obtained when females of the cancerous strains Z (C3H) and DBA from this laboratory were used as force breeders, and their data for mammary tumors were compared with females of the same strains that nursed their progeny (*17*).

Discussion of other pertinent data (*18–36*), including hormonal treatment, will be delayed until the observations have been considered from the present study in which mice of various groups, susceptible to the development of mammary cancer, with and without the mammary-tumor agent (MTA), were subjected to force breeding or the "functional test."

MATERIALS AND METHODS

Information concerning the strains of mice used in the present studies has been given previously (*17, 32, 37*). Animals of the cancerous Z (C3H) stock are included, together with mice of the agent-free, fostered Zb subline and the F_1 hybrids derived by reciprocal matings between mice of the Zb line and the agent-free Ax subline of strain A. No distinction will be made between the reciprocal hybrids, as the observations were similar: (AxZb)F_1 = Ax♀ × Zb♂ or (ZbAx)F_1 = Zb♀ × Ax♂.

Two litters of subline 2 of the cancerous strain DBA were obtained from Woolley in 1942. They had been inbred for either 32 or 33 generations since 1927 at which time brother × sister matings had been discontinued (*38*); later, Woolley (*39*) separated the mice into sublines. Inbreeding has been continued and members of the 70th generation were obtained during the past year.

The pregnant females usually were isolated in individual pens and those subjected to force breeding were returned to their breeding pen as soon as possible after their young were born. When they were born over a week end or holiday, they were not removed until the morning of the next working day. The normal breeders nursed their young until they were

large enough to wean, at approximately 4 weeks of age, or until they died from various causes.

The normal and force breeders of the respective stocks were observed during the same period. Because of space limitation, the majority of the agent-free females continued as normal breeders had to be killed after they had attained an age of 12 to 16 months, when it was necessary to replace the stock with young animals to provide mice for biological assay for the MTA. However, the force breeders were permitted to have as many litters as they would.

EXPERIMENTAL RESULTS

Normal and force-breeding females of sublines D2 and Z of the cancerous strains DBA and C3H are compared in tables 1 and 2, respectively.

TABLE 1.—Observations for mammary cancer in normal and force breeders of the cancerous D2 subline of strain DBA/2, tabulated according to the number of litters

Group	Litters	Number	Cancer (percent)	Average age (days)		Percent of total
				Cancer	Noncancer	
Normal breeders	1–2	10	70	298	501	3
	3–4	79	82	330	542	27
	5–6	144	67	380	529	49
	7–8	58	72	443	502	20
	9	3	67	466	455	1
	Total	294	72.4	375	524	
	Average No. litters			5.2	5.5	
Force breeders	1–2	11	73	371	422	19
	3–4	17	76	324	470	30
	5–6	21	81	369	419	37
	7–8	8	88	357	490	14
	Total	57	78.9	355	442	
	Average No. litters			4.6	4.9	

Although the force breeders of subline D2 showed a slightly higher incidence of mammary cancer than did the normal breeders, the reverse was seen for females of cancerous strain Z (C3H), but in no instance was the difference of statistical significance. The force breeders of Z (C3H) averaged more litters than did normal breeders, but the opposite was observed for females of D2. However, the force breeders of the latter gave rise to their tumors 20 days earlier than did the normal breeders (table 1), but no difference was apparent in females of Z (C3H) (table 2).

Only 40 percent of the normal breeders of strain Z (C3H) had 5 or more litters, while 75 percent of the force breeders had at least 5; in subline D2 the respective proportions were 70 and 51 percent. No correlation was observed in the percentage of the mice developing mammary cancer by age groups in the 2 series of females of these cancerous strains. In

TABLE 2.—Observations for mammary cancer in normal and force breeders of the cancerous strain Z (C3H), tabulated according to the number of litters

Group	Litters	Number	Cancer (percent)	Average age (days)		Percent of total
				Cancer	Noncancer	
Normal breeders	1–2	25	92	246	425	8
	3–4	158	96	253	379	52
	5–6	99	95	305	372	33
	7–8	21	67	345	454	7
	Total	303	93. 1	274	407	
	Average No. litters			4. 2	5. 0	
Force breeders	1–2	2	100	205	—	4
	3–4	10	100	237	—	21
	5–6	21	95	276	362	44
	7–8	15	73	296	320	31
	Total	48	89. 6	269	328	
	Average No. litters			5. 4	6. 8	

general, the ages at which the mice became cancerous were dependent upon the number of litters, the more litters the later the average cancer ages.

Observations for females of the agent-free Zb subline of strain Z (C3H) are recorded in table 3.

Four normal breeders of the Zb subline had mammary cancer and all were the progeny of female #265877, a member of the 45th generation since the line was fostered in 1938 (*32*). The mother, together with the ancestral females of each generation and her litter mates totaled 141 Zb females and remained free of mammary cancer after surviving to an average age of 457 days. The 4 cancerous Zb females were born in the first

TABLE 3.—Observations for mammary cancer in normal and force breeders of the fostered agent-free strain Z (C3H) or Zb, tabulated according to number of litters

Group	Litters	Number	Cancer (percent)	Average age (days)		Percent of total
				Cancer	Noncancer	
Normal breeders	3–4	98	3	188	460	17
	5–6	223	0. 4	247	448	40
	7–8	218	0	—	461	39
	9+	22	0	—	469	4
	Total	561	0. 7	203	456	
	Average No. litters			3. 3	6. 1	
Force breeders	1–2	2	0	—	565	7
	3–4	11	0	—	483	38
	5–6	5	0	—	473	17
	7–8	9	11	360	617	31
	9+	2	50	365	541	7
	Total	29	7	363	529	
	Average No. litters			8. 5	5. 2	

and second litters to female #265877. One female of the first litter had cancer when 247 days of age after bearing 5 litters of hybrids sired by a male of the agent-free Ax subline. Biological assay of the tumor demonstrated that it possessed the MTA, as do other tumors in this group. The source of the agent was unknown.

Of 29 Zb females which were continued as force breeders, 2, an incidence of 7 percent, developed mammary cancer (table 3). These mammary tumors were not tested for the MTA by biological assay. The two cancerous Zb females had 8 and 9 litters with a survival average of 363 days, compared with an average of 5.2 litters for the noncancerous force breeders that survived to an average of 529 days.

Of the normal breeders of the Zb subline, 83 percent had at least 5 litters and 43 percent had 7 or more; the percentages for the force breeders of the Zb subline were 55 and 38 percent, and again, the noncancerous normal breeders averaged more litters than did the noncancerous force breeders (table 3).

Information concerning the reciprocal (Zb $\times$ Ax)F_1 hybrids is recorded in table 4.

Over 900 F_1 hybrids were observed as normal breeders and only one, an incidence of 0.1 percent, had mammary cancer. This was noticed when the female was only 291 days of age, after bearing 5 litters. The tumor, an adenocarcinoma, was assayed and after 17 months the test animals have remained noncancerous, which indicates that the agent could not be demonstrated. Three sisters of the cancerous F_1 female were observed for an average of 439 days; each had 6 litters of young, and all died noncancerous. The noncancerous F_1 females had an average of 6.7 litters (table 4).

Only F_1 force breeders have been included that had at least 4 litters and were observed for 13 months or longer. One mammary tumor, an

TABLE 4.—Observations for mammary cancer in normal and force breeders of the F_1 generation produced by reciprocal matings between mice of the agent-free strain Ax and agent-free strain Zb

Group	Litters	Number	Cancer (percent)	Average age (days)		Percent of total
				Cancer	Noncancer	
Normal breeders	4	60	0	—	429	7
	5–6	316	0.3	291	429	35
	7–8	475	0	—	441	52
	9+	58	0	—	489	6
	Total	909	0.1	291	438	
	Average No. litters			5.0	6.7	
Force breeders	4	1	0	—	494	1
	5–6	11	0	—	731	13
	7–8	25	4	536	668	29
	9+	50	0	—	681	57
	Total	87	1	536	682	
	Average No. litters			7.0	8.9	

adenoacanthoma, was found in a hybrid when she was 536 days of age; the tumor was not assayed for the MTA. Noncancerous F_1 females lived to an average age of nearly 23 months and had 8.9 litters. Among the force breeders, 86 percent had 7 and more litters and 57 percent cast at least 9 litters. In the normal breeding group 59 percent had more than 7 litters and 6 percent had 9 or more litters. Many of this group were not maintained until the end of their breeding life but had to be replaced with younger animals because their progeny were needed for biological assays.

DISCUSSION

During the period when a maternal influence was being established as a causative factor in the genesis of mammary cancer in mice (*40*) and later demonstrated to be transferred in the milk of females of cancerous strains (*41–43*), Bagg (*2*) was investigating the effect of his "functional test" in relation to the same type of cancer in mice. The mothers were prevented from nursing their young by being returned to the breeding pens as soon as possible after their young were born. By subjecting young females to reproductive overwork, Bagg and his associates (*1*, *2*, *4–9*) reported that mammary cancer could be induced in females of the low cancerous C57BL stock. To account for their observation, the theory (*5*) was advanced that the prevention of nursing caused retention of milk because of improper drainage, and this in turn produced an irritating chemical effect upon the epithelium of the mammary cells.

Bagg had obtained animals of strain C57BL from the Jackson Memorial Laboratory, Bar Harbor, Maine, and later separated the mice into two sublines, depending upon the incidence of mammary cancer after they had been used in the functional test. Subline "Y" was selected toward a high incidence and substrain "A" toward a low incidence (*7*).

Little and Pearsons (*19*) could not corroborate Bagg's observations on the influence of force breeding upon the genesis of mammary cancer. They observed nearly 100 females of strain C57BL and, although some females had as many as 13 litters as force breeders, none developed mammary cancer.

In his 1936 publication (*2*), Bagg stated that the cancerous C57BL females had been reared by foster mothers. Personal communication (*44*) brought out the information that no record had been maintained concerning what female(s) had been used as the foster mother(s). It would seem probable that some foster mothers had been members of cancerous strains, possessing the mammary-tumor agent, and this they transferred to their fostered C57BL young. As a result, some secured the agent and later developed mammary cancer. This would explain why the females of strain C57BL observed by Little and Pearsons (*19*) remained noncancerous, as they had been nursed by their strain C57BL mothers without the milk-borne mammary-tumor agent.

Certain data available to Bagg by 1939 (*7*) showed that:

1) In mice of subline Y, the incidence of mammary cancer was as high in control, normal breeding females as in force breeders.

2) In mice of the so-called low-cancer subline A, the incidence of mammary cancer was as high when the young had been foster-nursed by females of cancerous strains as that observed in representatives of the so-called cancerous subline Y of strain C57BL. A high incidence was maintained in subline Y for 12 generations (*8*).

Andervont (*45*) found that mice descended from Bagg's strain C57BL remained low-cancerous, while an incidence of mammary cancer of 63 percent was found in C57BL females that had been fostered by females of the cancerous strain C3H/An.

In females of strain C57BL that obtained the MTA by nursing females of cancerous stocks, incidences higher than 70 percent have been reported by Fekete and Little (*46*) and Haagensen and Randall (*47*); data for other sublines have been reviewed in a recent publication (*11*). These high incidences were found in females permitted to nurse at least some of their litters, and equally high incidences were observed in their progeny or in females nursed by their strain C57BL mothers.

In 1936 Fekete and Green (*3*) studied the development of mammary cancer in females that had their nipples on the right side blocked with a red-hot wire while the experimental females nursed their young via the normal nipples on the opposite side. Again, females of strain C57BL remained noncancerous. Of their 49 experimental DBA females, 82 percent had mammary cancer. More tumors were found on the blocked than the unblocked side, and Fekete and Green concluded: "Blockage of the mammary ducts with resulting milk stagnation was influential in determining the site and the time at which mammary tumors appeared in a genetically susceptible strain, but did not itself cause tumors, since it was unable to overcome the hereditary resistance of a strain in which such tumor normally did not develop." Premature regression of the glands with blocked nipples was observed, though the secreting function of the glands appeared to be normal. No data were given for untreated controls.

Fekete and Green (*3*) included as neoplasms any nodule that was visible with the dissecting microscope in wholemounts of the mammary glands, even though the nodules were not palpable. This increased the number showing tumors in glands on both sides. It might be recalled that when only 4 glands were removed from cancerous *virgin* females of strain Z (C3H) and examined as wholemounts, the number of precancerous lesions per female ranged from 2 to as many as 66; several females without palpable nodules and tabulated as noncancerous had as many as 20 nodules per female (*48*). How many of these lesions could have been classified "cancerous" by any standard was problematical.

In addition, Fekete (*14*) subjected females of strains C57BL and DBA to other "functional tests." Regardless of their breeding history, females of strain C57BL remained noncancerous. When DBA females had lacta-

tion prolonged by the addition of young mice, 56 percent had cancer; others that gave birth to young in the breeding pen and were permitted to nurse had an incidence of 72 percent, whereas 87 percent of the females had tumors when they were allowed to nurse for only 1 day before being returned to the breeding pen. No difference of significance was apparent in the average cancer ages. The incidence of 87 percent in this series was higher than was observed in the previous group with blocked nipples (*3*).

In discussing the effect of force breeding upon the genesis of mammary cancer in DBA females, Fekete and Little (*46*) stated that the incidence was not increased as the result of this type of breeding.

Observations also have been reported by Mühlbock *et al.* (*49*, *50*) on the role of breeding, especially force breeding, upon the development of mammary cancer in DBA mice. Although biparous, force-bred females did not give a significantly higher incidence of mammary cancer than did virgins of the cancerous subline, with the respective incidences being 62 and 59 percent, tumors appeared earlier in mice of the former group. Force breeders with an average of 7 litters showed an incidence of 97 percent, yet normal breeders with an average of 6 litters had an incidence of only 68 percent (*50*).

Observations have been compared for virgin females, normal breeders, and force breeders of sublines D1, D2, and D8 of the cancerous DBA stock (*17*, reviewed in *11*). Nearly 600 animals were maintained in the various groups and in no instance did the force breeders of any cancerous line have a higher incidence of mammary cancer than did normal breeders of the same subline; in only 1 line did the force breeders have an earlier cancer age. The mean incidence for force breeders of the 3 sublines was 62 percent at 418 days, while the respective observations for normal breeders were 76 percent at 386 days. Contrasted with these data was the incidence of 59 percent in 183 virgin females, especially since the incidence found in nonbreeders of 2 sublines was higher than in force breeders of the same lines.

In the new material reported in table 1, the incidences for normal and force breeders of subline D2 were 72 and 79 percent; the cancer ages 375 and 355 days, respectively. Of the normal breeders, 49 percent had at least 5 litters of young, while only 37 percent of the force breeders had as many. The cancerous females of the respective groups averaged 5.2 and 4.6 litters.

For further evidence of subline differences in the DBA stock, it is interesting to make certain comparisons between the data reported by Chai and Russell (*33*), those observed by Mühlbock *et al.* (*49*, *50*), and those from this laboratory (*11*, *17*). While Chai and Russell did not maintain any females as force breeders, they found that the incidence of mammary cancer did not go above 50 percent in any subline until after the females had at least 4 litters; the mean incidence observed in virgin females by the others (*11*, *17*, *49*, *50*) was higher than this incidence.

Different effects of breeding were seen by Mühlbock *et al.* (*50*) and Andervont and Dunn (*23*) in agent-free DBA females. Only 1 of 96

breeders of an agent-free line of subline 212 observed by Andervont and Dunn (*23*) had cancer, yet the noncancerous females lived to an average age of 20 months. The increase in incidence seen by Mühlbock *et al.* (*50*) could have been influenced as much by an unequal distribution of mice by sublines, with their different incidences in agent-free animals, as by the effects of any particular method of breeding.

Subline differences within strain C3H, developed by Strong (*51*, *52*), have been recognized for many years. In 1941 Andervont reported that mice of his subline of strain C3H (*53*) should be regarded as a separate subline and could not serve as controls for data procured with animals of any other line of this inbred stock (*54*). The lines maintained by Heston (*26*, *55*), Mühlbock (*10*), and Deringer (*12*) are descended from the Andervont colony of strain C3H and are distinct from the Z (C3H) strain maintained in this laboratory (*16*, *17*, *32*, *40*, *56–58*). Amos (*59*) also has noted differences in some serologic characters among mice of the various sublines of strain C3H.

In a review of the data secured on the genesis of mammary cancer in mice of strain C3H, these subline differences must be kept in mind in any evaluation of the observations.

Three separate groups of force-breeding females of the cancerous Z (C3H) stock were studied and compared with normal-breeding females born during the same periods. These served as controls, and similar incidences and average cancer ages were observed for those in the 2 experiments. In the C3H force breeders, tumors appeared later in one group than in the controls, while in a more recent series the tumors appeared earlier. The average age for the 2 groups of force breeders, numbering 78 females, was similar to the normal nursing, or control females, but the incidence for the force breeders was only 81 percent compared with 94 percent in the controls (*17*).

In our investigations on the administration of tissue extracts to determine the activity of the MTA in bioassays, the greatest amount of lactating mammary tissue and milk could be secured from females that had nursed for approximately 12 days. Because Bagg (*5*) emphasized the role of stagnation of milk after force breeding, another group of C3H females was included in one experiment in which the C3H females were permitted to nurse their young for 12 days before they were returned to the breeding pen. Of 47 females observed, 74 percent had cancer at an average age of 263 days; the data for controls were 94 percent and 271 days (*17*).

In collaboration with Huseby, the young were removed from several C3H force breeders and the females were killed 24 hours later. Histological examination of their glands confirmed the gross findings that there was little milk production in the glands of strain C3H females that had not nursed their young and consequently little milk stagnation. However, those that had nursed for 12 days had greatly distended glands because of stagnation, and yet they had the lowest incidence of mammary cancer and no acceleration of the disease (*17*).

The data presented in table 2 on the development of mammary cancer in another group of force breeders of the cancerous Z (C3H) subline of strain C3H are in accord with the material published earlier (*17*). No significant difference was noted in the tumor incidences, though the higher incidence was seen in the normal breeders. While the force breeders averaged 5.4 litters compared with only 4.2 litters for the cancerous females of the control group, tumors did not develop earlier in the force breeders. Fifty-eight percent of the normal breeders had cancer after giving birth to only 4 litters, but only 25 percent of the non-nursing breeders had the disease after bearing the same number of litters.

Boot and Mühlbock (*10*) found that, while the appearance of mammary cancer could be accelerated from 255 to 209 days by force breeding females of their cancerous C3H subline, descended from Heston's line, the incidence was reduced from 93 to 82 percent. The females averaged 3.8 and 5.5 litters, respectively.

In animals originally descended from the Andervont subline of strain C3H, incidences ranging from 3 percent were seen in agent-free breeders by Andervont and Dunn (*23*) to 74 percent by Deringer (*12*), with intermediate incidences reported by Heston (*26*) and Boot and Mühlbock (*10*). Deringer found that the incidence increased from 55 percent in breeders that averaged 4.8 litters to 74 percent in force-bred, agent-free C3HeB females that averaged 6.1 litters. By maintaining mice descended from the C3H/An and C3H/He sublines in the same room and subjecting them to the same breeding system, Heston (*55*) demonstrated that there was no significant difference in either the tumor incidence or the average cancer age in agent-free females of the 2 sublines. Similar observations were made by Boot and Mühlbock (*10*) for females of their fostered subline and those descended from transferred ova to eliminate the MTA.

Heston (*55*) concluded "The mammary tumors occurring in these substrains had arisen, in the absence of the mammary-tumor agent, as a result of the high genetic susceptibility, the hormonal stimulation from breeding, and the advanced ages of the females." Also, "There was a positive correlation between the number of litters these C3Hf females produced and the incidence of mammary tumors. The incidences ranged from 2 percent in virgin females to 44 percent in females that had 7 litters each."

Regardless of these relatively high incidences in agent-free females of strains C3H/An and C3H/He, only 2 of 29 of the agent-free Zb females had mammary cancer after they were used as force breeders, an incidence of 7 percent, compared with an incidence of 0.7 percent for the normal breeders. The noncancerous normal breeders averaged 6.1 litters as contrasted with 5.2 cast by the force breeders. The average number of litters born to representatives of our subline of strain C3H was nearly the same as the number cast by females of substrains C3H/He in which the incidences were significantly higher (*10, 12, 26, 55*). Also, the average cancer age observed by Deringer (*12*) in agent-free C3H/He force-bred females of 537 days compared with the noncancer age of our force-bred

Zb females of 529 days (table 3). This would be in accord with the 1941 statement by Andervont (*54*) that experimental data obtained with mice of one subline may not be controlled by animals of our subline of strain C3H.

Since 1943, Andervont and Dunn (*20, 23–28, 30, 45*) have made crosses in which representatives of strain C3H/An were crossed with mice of other inbred stocks and have observed the incidence of mammary cancer in the F_1 progeny. In the crosses between strain C (BALB/c) females and males of strain C3H/An, high incidences have been found in F_1 breeding females, yet no significant difference was seen in the incidence in F_1 progeny sired by either agent-containing or agent-free strain C3H/An males except where there was male transmission of the MTA (*30*).

The high incidence observed in the (C♀ × C3H/An♂)F_1 hybrids has been confirmed in this laboratory, but when males of our subline Z (C3H) were used, the F_1 progeny born to noninfected C mothers had an incidence of only 5 percent with an average cancer age of 654 days (*11, 32, 34, 37*). As was observed in mice of the agent-free Zb subline (table 3), force breeding did not increase the incidence of mammary cancer in F_1 females of the reciprocal cross between mice of strains Ax and Zb, in which the noncancerous F_1 females averaged 8.9 litters and survived to an average age of nearly 23 months.

Mice of various sublines of strain C3H and their F_1 hybrids have received considerable attention in their response after hormonal treatment, either by the implantation of pellets subcutaneously or by the addition of the hormone to drinking water (*10, 12, 26, 31, 36, 60, 61*). In general, the incidence seen in hormone-treated animals was comparable to that observed after force breeding, though Boot and Mühlbock (*10*) found the highest incidence in castrated males of their agent-free C3H subline after high-dose estrogen treatment.

As with force breeding, agent-free mice of the Zb subline failed to develop tumors when a small number was subjected to hormone therapy (*61*).

Andervont, Dunn, and Canter (*36*) implanted diethylstilbestrol-cholesterol pellets subcutaneously in agent-free animals of strain C3H/An and found that a higher incidence of mammary tumors was induced in castrated males than in females receiving similar pellets. The authors concluded that the mammary-tumor agent accelerated the production of mammary tumors in mice possessing a tendency toward the development of these tumors when the mice were exposed to hormonal stimulation.

In discussing the genesis of different types of mammary cancer in mice, Andervont, Dunn, and Canter (*36*) concluded: "When these different modes of development for mammary tumors in mice are considered, it is evident that the histogenesis of the tumors present the same diversity as was found in the morphologic pattern of the tumors themselves, and that the modes of origin are probably no more uniform than are the resultant tumors. These differences in histogenesis have been related to the inbred strain, age, sex, presence of the agent, and hormonal treatment. All these

factors should be considered when either the histogenesis or the morphology of mammary tumors is described, and it is not surprising that different investigators have observed different modes of origin of mammary tumors in mice."

The data reported by many investigators demonstrate that subline differences within inbred strains may be important in influencing the incidence and average age of mammary cancer found in virgins, normal breeders, and others subjected to force breeding. These and other factors, such as degree of hormonal stimulation, must be considered in any interpretation of the data.

The new observations considered in this manuscript are in accord with those obtained by Boot and Mühlbock (*10*) and from this laboratory (*17*) that force breeding need not increase the incidence of mammary cancer in C3H and DBA females. Likewise, there need be no correlation between the time of development of cancer and the number of litters cast by the normal and force breeders of cancerous strains.

In agent-free females of the Zb subline and in F_1 females obtained by reciprocal matings between animals of the agent-free sublines of strains A and Z (C3H), or Ax and Zb, force breeding did not significantly increase the incidence of mammary cancer. In this respect, females of the Zb subline differed from those of either strains C3H/An or C3H/He and their substrains (*10–12, 23, 26, 34, 37, 55*).

In addition, these observations on force-bred females would not support the theory suggested by Bagg (*5*) that prevention of nursing resulted in stagnation of the mammary ducts, and that this contributed to the development and/or acceleration of mammary cancer. In agent-free females, comparable observations were obtained after subjection of the females to the functional test and excessive hormonal stimulation (*10, 12, 26, 31, 55, 60, 61*). Thus, any influence of force breeding upon the genesis of mammary cancer may result from increased hormonal stimulation correlated with breeding and lactation, especially in the younger animals.

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Enzymatic Deamination of Deoxyadenylic and Adenylic Acids by Normal and Cancerous Rat Liver Tissues [1, 2, 3]

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SUMMARY

To obtain information of the possible role of the enzymatic deamination of 5′-deoxyadenylic (dAMP) and 5′-adenylic acids (AMP) in rat liver carcinogenesis, the process was studied by a combination of polarographic, spectrophotometric, and paper chromatographic methods on homogenates and isolated subcellular fractions of normal rat liver, cancerous liver, and Novikoff tumor. Mouse Sarcoma 180 was also included. Whereas the homogenates quickly deaminated both AMP and dAMP in all cases, none of the purified subcellular particulate fractions alone could deaminate these nucleotides. The combination of supernatant and cytoplasmic elements (mitochondria and microsomes) did deaminate AMP and dAMP. 5′-Deoxyinosinic acid (dIMP) or 5′-inosinic acid (IMP) was never found in liver or tumor preparations while deoxyadenosine (dA), deoxyinosine (dI), hypoxanthine (in dAMP deamination) and inosine (I), hypoxanthine (in AMP deamination) were detected. There was a sufficient parallel between dephosphorylation and deamination of both nucleotides to warrant the conclusion that the enzymatic deaminations of AMP and dAMP in rat liver and liver tumors are brought about by the action of two enzymes: a phosphatase in the subcellular granules and adenosine (A) deaminase in the supernatant. The reactions are dAMP→dA→dI and AMP→A→I. The more rapid deamination of both nucleotides by tumor tissue is not due to increased levels of nucleotide-deaminase activity in tumors, but solely to the greater cell number per unit of weight in tumor as against corresponding normal tissue. In 3′-methyl-4-dimethylaminoazobenzene (3′-Me-4-DAB) carcinogenesis, however, the increased rate of A deamination was found to be a reversible phenomenon pertaining to the metabolism of the dye.—J. Nat. Cancer Inst. 26: 1059–1073, 1961.

IN CONTRAST to normal tissue in which a balance between the processes of growth and tissue breakdown is homeostatically regulated within the

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[3] Abbreviations used in text: AMP: 5′-adenylic acid; dAMP: 5′-deoxyadenylic acid; IMP: 5′-inosinic acid; dIMP: 5′-deoxyinosinic acid; A: adenosine; I: inosine; dA: deoxyadenosine; dI: deoxyinosine; DNA: deoxyribonucleic acid; RNA: ribonucleic acid; TCA: trichloroacetic acid; PCA: perchloric acid; 3′-Me-4-DAB: 3′-methyl-4-dimethylaminoazobenzene (*Chemical Abstracts' Nomenclature: N, N*-dimethyl-*p*-phenylazoaniline).

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organism, the unrestricted proliferation of tumors indicates an irreversible shift in favor of anabolic, growth-promoting reactions. The reactions governing growth and thus primarily protein synthesis are linked to the synthesis of the nucleic acids. Evidence has been obtained recently for a substantial decrease in tumors of several enzymes that catabolize purines or their ribosides (*1*), together with the depletion of 5′-nucleotidase (*2*) and the complete disappearance of xanthine oxidase (*3*, *4*). These factors may increase the intracellular concentration of the precursors in ribonucleic acid synthesis.

The depletion of deoxynucleotidases, the enzymes which liberate inorganic phosphate from deoxynucleotides and convert them to the corresponding deoxynucleosides, was described recently (*5*, *6*) and indicated that the depletion of at least some catabolic enzymes connected with deoxyribonucleic acid metabolism is also to be found in rat liver carcinogenesis.

More significant for the elucidation of the mechanism of tumor growth than the various enzymatic depletions are the activations of anabolic processes connected with the metabolism of nucleic acids, since these would inform us more directly about specific steps actually taken by the cell in the activation of RNA and DNA synthesis. Thus, the recent observation of a high activity of deoxycytidylic acid deaminase in certain tumors (*7*, *8*) points to deoxyuridylic acid as a transient precursor in the DNA synthesis of these tissues, by the methylation of deoxyuridylic acid to thymidylic acid (*9*). The recent reports (*10*, *11*) that the levels of adenylic acid deaminase and adenosine deaminase are highly increased in primary liver tumors, Novikoff tumor, and even as an early effect of carcinogen feeding, if confirmed, would be of similar interest. Indeed, it was concluded (*10*, *11*) on the basis of these observations that an increased formation of inosinic acid in tumors may facilitate the conversion between adenine and guanine compounds, and thus indirectly the RNA synthesis in tumors.

This study was undertaken primarily to obtain information concerning the enzymatic deamination of deoxyadenylic acid by normal and cancerous rat liver tissues. AMP deaminase is known (*12*) to deaminate dAMP only to a very small extent, so that observations analogous to those reported for AMP (*10*, *11*) could not be expected *a priori*. Moreover, it was thought that in the observations mentioned (*10*, *11*) the formation of IMP had not been proved experimentally, so that the action of AMP deaminase in tumor tissue and its increase in rat liver carcinogenesis was by no means established. The data will show, in fact, that in rat liver and liver tumors both AMP and dAMP are enzymatically deaminated, not by a specific nucleotide deaminase, but by the combined effects of a phosphatase in the microsomes (AMP) or in the mitochondria (dAMP) and adenosine deaminase in the supernatant.

METHODS

Preparation of tissue and cell fractionation.—Adult male Sprague-Dawley rats were fed commercial pellets or basal semisynthetic, carbohydrate-rich, riboflavine poor diet with or without (controls) carcinogenic 3′-methyl-4-dimethylaminoazobenzene in 0.06 percent concentration. Either transplantable Novikoff tumors ("hepatoma") or primary liver tumors, induced in about 200 days of carcinogen feeding, were used. Tissue from thigh muscles of mice and mouse Sarcoma 180[5] were also used for comparison. The tissue (6–20%) was homogenized in a glass homogenizer with ice-cold isotonic (0.25 M) sucrose or 0.2 M tris(hydroxymethyl)aminomethane buffer of *p*H 7.5, the isotonic sucrose being used when fractionation of the homogenate was intended. Subcellular fractions were obtained by conventional methods of differential centrifugation with the use of a refrigerated International centrifuge for spinning down the nuclei (600 × *g*, 10′) and the Servall SS-1 centrifuge for spinning down the mitochondria (20,000 × *g*, 7′). The microsomal fraction was obtained by centrifugation for 1 hour in the refrigerated Spinco Model L preparative ultracentrifuge at 59,000 × *g* (25,000 rpm, with use of the rotor #30). When it was intended to separate the homogenate into soluble and insoluble fractions only, the homogenate was directly centrifuged for 1 hour at 25,000 rpm in the Spinco ultracentrifuge, and the sediment obtained was resuspended in sucrose and recentrifuged at the same speed for 1 hour. Isolated fractions were used after being washed in the original volume of sucrose, sedimented by recentrifugation, and resuspended in tris buffer.

Chemical determinations.—Commercial preparations of dAMP and dA were purchased from the Sigma Chemical Company, 3500 Dekalb Street, St. Louis, Missouri. AMP, in the form of the sodium salt, IMP, and inosine were obtained from Pabst Laboratories, 1037 West McKinley Avenue, Milwaukee, Wisconsin. Adenosine was purchased from Schwarz Laboratories, Inc., 230 Washington Street, Mount Vernon, New York. The dAMP was neutralized in water solution with NaOH and diluted with tris buffer to the desired concentration. All substrates were found pure by paper chromatography.

Unless specified differently, the homogenate or separated subcellular fraction (0.1–1.0 ml) was incubated in duplicate with 1.0 ml of substrate (3–5 μmoles of dAMP, AMP, dA, or A) and 2.0 ml of 0.2 M tris buffer (*p*H 7.5) at 37.8° C for various periods. In one set of tubes the reaction was stopped by the addition of 5.0 ml of 5 percent perchloric acid, while to the duplicate set 1.0 ml of 30 percent trichloroacetic acid was added. This arrangement was used to perform polarographic and spectrophotometric measurements in perchloric acid extracts (one half of which was also used for paper chromatography) and a parallel determination of the inorganic phosphate in TCA extracts, which are not directly suitable for

[5] Obtained through the courtesy of Dr. Samuel Graff and Mr. Gerald Zagall, Department of Biochemistry, Columbia University, New York, N.Y.

either polarography or spectrophotometry. The TCA extraction was performed twice, the second time with 5 percent TCA, so that the combined supernatants after centrifugation totaled 9.0 ml. Inorganic phosphate was determined by King's modification (*13*) of the Fiske and Subba Row method.

The deamination of adenine compounds was followed polarographically on the PCA extracts.[6] As shown by Heath (*14*), only the adenine derivatives among the purine compounds and none of the pyrimidines are reducible on the dropping mercury electrode. In 5 percent PCA the adenine derivatives give a characteristic wave at −1.05 volts against the saturated calomel electrode, the height of the wave being strictly proportional to the concentration. The method is extremely rapid, unusually suited for kinetic studies on the enzymatic deamination of adenine compounds. It is superior to the colorimetric determination of liberated ammonia in that it is quite specific, can be performed directly on PCA extracts, and its sensitivity is such that, after the elimination of oxygen from the solution, we could identify adenine base in the eluate (50 μl) from a single spot on our chromatograms. However, the adenine wave in PCA extracts of normal liver can be obscured by a catalytic increase of the current from an undisclosed origin which occurs at nearly the same potential as the reduction of adenine. This complication may be circumvented by dialysis of the liver homogenate or fraction for 24 hours against normal saline and by a decrease in the speed of polarographic recording. The polarograph used in the experiments was the KLB-Blomgren type 3266, attached to a Leeds-Northrup automatic recorder (Speedomax type G). For most purposes it was sufficient to perform polarographic determinations in beakers (10–15 ml) open to air. Curves were usually recorded from a starting potential of −0.8 volts, with a sensitivity of 20–100 μa for full-scale deflection.

Spectrophotometric data, with the use of the Beckman Model DU spectrophotometer, were obtained as the differences between the optical densities of diluted (5%) PCA extracts (final vol. 100 ml) of samples incubated with and without substrate. At the *p*H of 5 percent PCA (*p*H$<$0), the absorption maximum of adenine compounds is at 257 mμ, that of inosine compounds at 248 mμ. The ratio of optical densities at these or nearby wavelengths varies as a function of the amount of deaminated products. In most reactions with homogenate or supernatant, however, hypoxanthine was formed along with the inosinic compounds, and since the molar absorptions of these compounds differ from each other and from the molar absorption of adenosine, it was impossible to evaluate quantitatively the rates of deamination from spectrophotometric data alone. The ratios E_{260}/E_{248} merely gave qualitative estimations of the extent of deamination, while the exact amount of deaminated substrate was determined polarographically.

Paper chromatography.—Substrates and products in each type of re-

[6] The feasibility of the polarographic method for enzymatic assay in these experiments was suggested by Mr. Emerich S. Fiala, who also performed the first determinations in this laboratory.

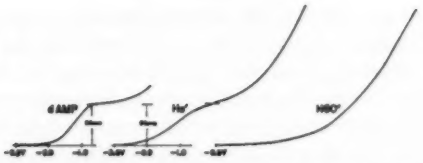
action performed were checked by descending chromatography on Whatman paper #3MM in a closed cabinet ("Chromatocab"). Because the low *p*H of PCA extracts (but not of TCA extracts) may considerably lower the yield of deoxynucleotides or even dephosphorylate them completely, the PCA extracts when used for chromatography were immediately neutralized with KOH at near zero temperature and, after the $KClO_4$ precipitate was centrifuged off, the neutral supernatants were either placed on the paper immediately or lyophilized and stored at −18° C. The material was then dissolved in a small volume (2–3 ml) of distilled water, the undissolved portion being centrifuged off. One hundred to 200 µl of sample were used for each spot, usually in duplicate or triplicate. The solvent consisted of a mixture of ammonium acetate, ammonia, ethanol, and Na tetraborate (*15*),[7] *p*H 10. In some tests a two-dimensional run was performed with the mixture of ethanol, ammonia, and ammonium acetate, *p*H 7.5 (*16*), preceding the *p*H-10 solvent. The two-dimensional run was no more advantageous than the one-dimensional chromatography for, although the separation of nucleotides from nucleosides was equally successful in both methods, the separation of AMP from IMP did not occur in either solvent. The R_f values of UV-absorbing spots with the use of *p*H-10 solvent were: AMP and IMP, 0.1; I, 0.3; A, 0.35; hypoxanthine, 0.57; dIMP, 0.24; dAMP, 0.32; dI, 0.68; dA, 0.74. We identified these compounds by comparing the R_f values with the R_f's of commercial preparations and by spectroscopic tests of the eluates, together with the *in situ* reaction of cysteine-sulfuric acid on deoxyribose compounds (*17*) and the ammonium molybdate test of Hanes and Isherwood (*18*) on nucleotide phosphate. In the case of dIMP, no commercial sample was available for comparison of the R_f values. On the basis of positive deoxyribose and phosphate tests (*17*, *18*), however, and since the eluant gave an absorption spectrum characteristic of an inosinic compound, the spot having R_f 0.24 was identified as dIMP.

RESULTS

Enzymatic Deamination of AMP and dAMP by Muscle Tissue and by Mouse Sarcoma 180

To obtain information from conditions in a tissue known to be a rich source of AMP deaminase, the activity of this enzyme from thigh muscles of mice was compared to the activity in mouse Sarcoma 180. A similar comparison also was made of the enzymatic deamination of dAMP in both tissues. Polarographically, a rapid and complete deamination of both AMP and dAMP by homogenates of sarcoma and muscle tissue was observed. Text-figure 1 illustrates the previously unrecorded deamination of dAMP by muscle and indicates the scales of the potential and current intensity used in the polarographic determinations.

[7] In these solutions we substituted Na tetraborate for the "borate" of Plesner.



TEXT-FIGURE 1.—Polarographic detection of dAMP deamination by the homogenate of mouse muscle tissue. Curve 1: 5 μmoles dAMP in 9.0 ml 5 percent $HClO_4$. Curve 2: dAMP + 200 mg tissue, 0′ incubation. Curve 3: *Id.*, 60′ incubation. Sensitivity: 200 μa.

Spectrophotometrically, both tissues behaved alike in their action on AMP and dAMP; after 1 hour's incubation, the spectrum of adenylic compounds changed to the spectrum of inosinic compounds. Only paper chromatography could detect the difference between the enzymatic deamination of AMP and that of dAMP by muscle and by Sarcoma 180, as illustrated in table 1.

TABLE 1.—Chromatographic separation of deamination products*†

	AMP				dAMP			
	Muscle		Sarcoma 180		Muscle		Sarcoma 180	
	0′	60′	0′	60′	0′	60′	0′	60′
R_F	0.1(v.s.)	0.1(v.s.)	0.1(v.s.)	—	—	0.24(s)	—	—
	—	0.3(w)	—	0.3 (s)	0.3(s)	—	0.3(s)	—
	—	0.57(w)	—	0.57(s)	—	0.58(s)	—	0.58–0.6(s)
	—	0.69(v.w.)	—	—	—	—	—	0.68–0.72(w)

*Spots were eluted with 4 ml 5 percent $HClO_4$, their UV spectrums measured, and the amounts calculated from molar absorption coefficients; μm = micromoles actually present in the spot. The intensity of each spot as seen under UV light is abbreviated as follows: s = strong; w = weak; v.s. = very strong; v.w. = very weak.

†On the basis of the identity tests mentioned, the chromatogram reads as follows: muscle: 0′ = AMP (0.1 μm); 60′ = IMP (0.08 μm); I (0.01 μm); hypoxanthine (0.03 μm), and an unidentified compound seen as a very weak, white, fluorescent spot (R_F 0.69); sarcoma: 0′ = AMP (0.1 μm); 60′ = I (0.044 μm); hypoxanthine (0.062 μm); muscle: 0′ = dAMP (0.1 μm); 60′ = dIMP (0.05 μm); hypoxanthine (0.05 μm); sarcoma: 0′ = dAMP (0.1 μm); 60′ = hypoxanthine (0.0445 μm); dI (0.052 μm).

The formation of dIMP by muscle tissue was surprising and it has not been decided at this time whether dIMP was formed unspecifically by a tremendous activity of AMP deaminase in the muscle or by the specific activity of another enzyme in the tissue. Relevant to our problem, however, was the fact that neither IMP nor dIMP was formed by Sarcoma 180, the only deamination products being inosine (deoxyinosine) and hypoxanthine. The possibility that IMP or dIMP could have been dephosphorylated during the incubation period was excluded by experiments in which an excess of substrate or shorter incubation periods did not result in complete dephosphorylation of the nucleotide, yet no trace of IMP or dIMP could be detected. In such cases, enough of the excess substrate would have remained to be acted upon by AMP deaminase or dAMP deaminase, if these enzymes were present at all. However, the

eluates from the nucleotide spots spectrophotometrically displayed the spectrum of a pure adenylic compound without the shift in absorption toward 248 mμ that characterizes an inosinic compound.

Enzymatic Deamination of AMP and dAMP by Normal Rat Liver and Novikoff Tumor

In rat liver tissue the enzyme AMP deaminase has been variously described as present in microsomes (*19*), in the soluble phase (*20*), or undetectable in any subcellular liver fraction (*21*). We observed that all subcellular liver fractions deaminated AMP, but only when the fractions were unpurified. In contrast to the whole homogenate, none of the purified particulate subcellular fractions (nuclei, mitochondria, and ergastoplasm) was able to deaminate either AMP or dAMP. The supernatant (1 hr at 59,000 × *g*) deaminated these nucleotides when not deprived of cytoplasmic particles by prolonged centrifugation. Purification of the supernatant (2 hrs at 20,000 × *g* plus 1 hr at 59,000 × *g*) almost completely removed this activity, although the purified supernatant rapidly deaminated A and dA to I and dI, respectively. Combining purified supernatant with particles initiated or increased the rate of AMP and dAMP deamination.

Paper chromatograms for normal rat liver with the use of the ammonium-acetate, ammonia-ethanol-Na tetraborate system showed that besides dA (R_F 0.74), the only products of dAMP deamination were dI (R_F 0.68) and hypoxanthine (R_F 0.57). No spot corresponding to dIMP was found. In the AMP deamination, no IMP but only I (R_F 0.3) and hypoxanthine were detected.

That AMP or dAMP deaminase could not be detected in any single subcellular fraction and that spots corresponding to IMP and dIMP were missing from paper chromatograms led us to the explanation that the deamination was the result of the combined effects of a phosphatase and adenosine deaminase, the phosphatase (5′-nucleotidase) being localized (*2*) in the nuclei and microsomal fractions. This explanation was experimentally tested as follows: 10 g of liver tissue was homogenized in 60 ml isotonic sucrose and the homogenate centrifuged for 1 hr at 20,000 × *g*. The supernatant ("S_1") was divided into 2 parts, one of which was centrifuged for 2 more hrs at 59,000 × *g* ("S_2"). After incubation with AMP, both supernatants liberated inorganic phosphate which, as indicated in table 2, went parallel to the deamination in both cases. The removal of microsomes by prolonged centrifugation at a high speed decreased the rate of AMP deamination to the extent that the phosphatase had been removed from the supernatant, and thus confirmed the explanation that both a phosphatase in the microsomes and adenosine deaminase were involved in the deamination.

Table 2 shows also that similar results were observed for the deamination of dAMP by normal rat liver, though in this instance the phosphatase, a deoxynucleotidase, differed from 5′-nucleotidase and has been localized

TABLE 2.—Correlation between dephosphorylation and deamination of AMP and dAMP by supernatant fraction of normal rat liver*

Subtrate	Fraction	Substrate initially present (μmoles) †	Substrate deaminated after 60′ (μmoles) ‡	Conversion (percent)	Inorganic phosphate liberated (μmoles)	Substrate dephosphorylated (percent)
AMP	S_1	3. 64	2. 9	80. 0	2. 38	65. 5
	S_2	3. 64	2. 0	57. 0	1. 58	43. 5
dAMP	S_1	4. 55	3. 64	80. 0	3. 65	80. 0
	S_2	4. 55	1. 37	30. 0	1. 30	28. 5

*Incubated mixture: supernatants (S_1 and S_2) from 200 mg tissue + 1 ml substrate + 2.0 ml tris buffer.
†Calculated on spectrophotometric basis.
‡On spectrophotometric and polarographic basis.

(*5*) predominantly in the mitochondrial fraction. For dAMP, however, the correspondence between deamination and liberation of inorganic phosphate was completely quantitative. The greater effect resulting from prolonged centrifugation than in the AMP deamination was due to the presence of the deoxynucleotidase dephosphorylating dAMP in the heavier subcellular elements, the mitochondria, whereas 5′-nucleotidase, present in the much lighter microsomes, could not be removed completely from the supernatant after centrifugation for 1 hour at 59,000 × *g*.

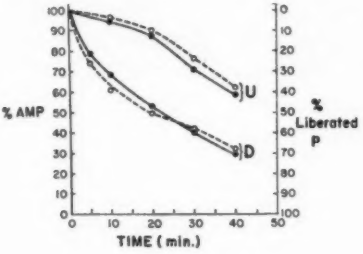
Similar experiments with the Novikoff tumor in place of the rat liver fractions (*see* table 3) showed that tumor tissue acted analogously to the normal tissue in the deamination of both AMP and dAMP. The quantitatively imperfect agreement between dephosphorylation and deamination with AMP, which is illustrated in table 2, was probably due to the

TABLE 3.—Correlation between dephosphorylation and deamination of AMP and dAMP by homogenized Novikoff tumors*

Substrate	Fraction	Substrate initially present† (μmoles)	Substrate deaminated‡ (μmoles)	Conversion (percent)	Inorganic phosphate liberated (μmoles)	Substrate dephosphorylated (percent)
AMP (15′)§	Whole homogenate	5. 08	0. 86	17. 0	0. 73	14. 0
AMP (60′)	Sediment	5. 2	0. 81	15. 5	2. 24	42. 6
	Supernatant	5. 2	1. 67	32. 0	2. 03	39. 0
	Sediment + supernatant	5. 2	5. 2	100. 0	5. 20	100. 0
dAMP (60′)	Whole homogenate	4. 06	4. 06	100. 0	3. 95	97. 0
	Sediment	4. 12	0. 25	6. 0	2. 32	56. 0
	Supernatant	4. 12	1. 32	32. 0	1. 38	33. 0
	Sediment + supernatant	4. 12	2. 68	65. 0	2. 38	58. 0

*Incubated mixture: 1.0 ml fraction (whole homogenate from 100 mg of tissue, supernatant, sediment, supernatant + sediment) + 1.0 ml substrate + 2.0 ml tris buffer.
†Spectrophotometrically determined.
‡Polarographically determined.
§Incubation time.

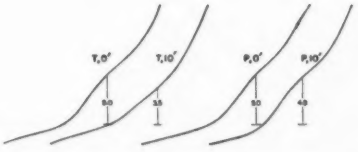
inability to determine exactly the inflection point of polarographic voltage-current curves when undialyzed liver tissue was tested. For this reason we reinvestigated the same reaction, using dialyzed Novikoff tumor homogenate and much shorter incubation times. As shown in text-figure 2, an almost perfect quantitative correlation between



TEXT-FIGURE 2.—Correlation between dephosphorylation and deamination of AMP by dialyzed Novikoff tumor homogenate. AMP: 1 μmole; amount of tissue: 40 mg wet weight; U: undialyzed; D: dialyzed; *full lines*: liberation of inorganic phosphate; *broken lines*: polarographic determinations of AMP.

dephosphorylation and deamination of AMP was found. When the undialyzed portion of the homogenate was tested, the rate of both dephosphorylation and deamination was much slower as compared to the dialyzed homogenate, especially during the first 10′ of incubation; in both cases, however, there was a complete quantitative agreement between dephosphorylation and deamination.

The lower rate of both reactions (dephosphorylation and deamination) in the undialyzed homogenate was tentatively explained as the inhibition of deamination by the high concentration of inorganic phosphate before dialysis. To see whether this explanation was correct, the dialyzed homogenate was assayed with AMP in two parallel tests, the first with tris buffer in the incubation mixture, the second with phosphate (0.1 M) buffer of the same *p*H (7.3). After 10′ the PCA extracts were examined polarographically and spectrophotometrically. Text-figure 3 shows that

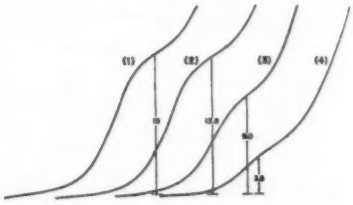


TEXT-FIGURE 3.—Inhibition of AMP deamination by inorganic phosphate. Curves 1 and 2: deamination in tris buffer (0.2 M), 0′ and 10′; curves 3 and 4: deamination in phosphate buffer (0.2 M), 0′ and 10′; AMP: 1 μmole; amount of tissue: 40 mg wet weight; sensitivity: 20 μa.

incubation in phosphate buffer led to an almost complete inhibition of the deamination, whereas in tris buffer the deamination was about 50 percent. Spectrophotometrically, almost no change in the absorption spectra could be detected in extracts from the phosphate medium, while a pronounced shift toward 248 mμ occurred in the extract from the tris buffer medium. This, therefore, is the final proof that the phosphatase action is needed in order that deamination of AMP may proceed, since it is known (*22*) that inorganic phosphate inhibits 5′-nucleotidase, whereas it does not inhibit either AMP deaminase or A deaminase. A similar

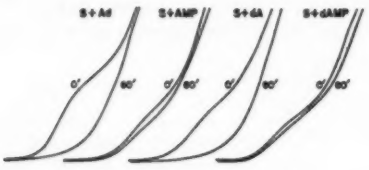
test with sodium fluoride would not succeed because fluoride is known to inhibit both 5′-nucleotidase and AMP deaminase.

While homogenates of Novikoff tumor rapidly deaminated both nucleotides, the sediment, obtained by centrifugation for 1 hour at 59,000 × *g* with washing and resuspension, did not deaminate either AMP or dAMP. Purified supernatant showed slight activity, but the combination of inactive sediment with slightly active supernatant resulted in a large increase in both AMP and dAMP deamination. Text-figure 4 shows a typical experiment illustrating the enzymatic deamination of dAMP by Novikoff tumor fractions as measured by the polarographic method.



TEXT-FIGURE 4.—Enzymatic deamination of dAMP by Novikoff "hepatoma." Incubated mixture: 1.0 ml fraction [whole homogenate from 200 mg of tissue, supernatant (2), sediment (3), supernatant and sediment (4)] + 5.2 μmoles dAMP + tris buffer. Total volume: 4.0 ml. Time: 60 minutes; curve (1) represents 0 minutes control with homogenate. Each wave started from −0.8 volts. Sensitivity of 100 mμ for full-scale deflection. *See* text-figure 1 for scale of potential and current intensity.

Text-figure 4 shows that the supernatant in the experiment described deaminated dAMP to some extent. This prompted us to test the activity in a supernatant obtained by prolonged centrifugation (2 hrs at 20,000 × *g* plus 2 hrs at 59,000 × *g*). Text-figure 5 indicates that, as in the purified



TEXT-FIGURE 5.—Enzymatic deamination of several adenine derivatives by supernatant from Novikoff tumor. AMP, dAMP, A, dA = 5 μmoles.

supernatant ("S_2") from normal rat liver, this step removed most of the ability of the supernatant to deaminate AMP or dAMP, while both A and dA could still be deaminated.

Enzymatic Deamination of A and dA by Normal and Cancerous Liver Tissue and Novikoff Tumor

The rapid deamination of A and dA by normal rat liver, Novikoff tumor, and mouse Sarcoma 180 contrasts with the absence of AMP deaminase in these tissues. From the reported depletions of 5′-nucleotidase (*2*) and deoxynucleotidase (*5*) in tumors, one might expect this effect to be linked to a relatively high content of A and dA deaminases.

Table 4 shows, however, that, although Novikoff tumor is more active than normal rat liver in both A and dA deamination on a wet-weight basis, the tumor actually displays less activity than normal liver on a per-cell or unit of DNA basis. This effect is due to the fact that Novikoff tumor has approximately 2.6 times the cell number per unit weight of tissue than does the corresponding normal tissue.

TABLE 4.—Enzymatic deamination of A and dA*

			Substrate present (μg) †	Substrate deaminated (μg)	DNA (μg)	DNA units (5×10^{-12}g) in millions	μg Substrate deaminated/million units DNA
A	Normal rat liver	0′	955	0	78	15.6	0
		15′	746	209			13.4
		30′	628	327			21.0
	Novikoff tumor	0′	955	0	207	41.6	0
		15′	679	276			6.6
		30′	424	531			12.8
dA	Normal rat liver	0′	825	0	78	15.6	0
		15′	581	244			15.7
		30′	225	600			38.5
	Novikoff tumor	0′	825	0	207	41.6	0
		15′	545	280			6.7
		30′	221	604			14.5

*Incubated mixture: homogenate 0.5 ml (100 mg wet weight), tris buffer 1.5 ml, substrate 1.0 ml.
†Determined polarographically.

In carcinogenesis induced by 3′-Me-4-DAB, however, an increase in the rate of A deamination in rat liver was observed, in agreement with the observations of Chan *et al.* (*10*, *11*) and other workers (*1*), as long as the experimental animals were killed while on the carcinogenic diet. The data in table 5 show that the A deaminase activity increased about 100 percent in 56 days after the initiation of carcinogen feeding even on a per-cell basis. A small increase (33%) in the rate of A deamination was observed in the liver homogenates of rats fed carcinogen for 204 days when tumor nodules were present in the grossly enlarged liver. This finding seemed to contrast with the depletion, on a per-cell basis, of A deaminase in Novikoff tumor, so that its significance was at first not clear. It was subsequently found, however, that the increase in the rate of A deamination during feeding with carcinogenic azo dye was a reversible phenomenon, which disappeared when the animals were put back on basal diet several days prior to being killed. It appears, therefore, that the increased rate of A deamination is not a permanent feature of malignant transformation, but pertains rather to the metabolism of the carcinogen 3′-Me-4-DAB.[8]

[8] This possibility was brought to our attention by a comment of a reviewer of this paper.

TABLE 5.—Enzymatic deamination of A in rat liver during experimental carcinogenesis*

		Amount of tissue (mg)	A at 0′ (μg)	A deaminated in 30′ (μg)	DNA (μg)	DNA units (5 × 10^{-12}g) in millions	μg A deaminated/million units DNA	Remarks
56 Days	Controls (2)	52	2000	780	150	30.0	26.0	Basal diet (B.D.)
	Experimental (2)	54	2000	1312	138	27.2	48.5	B.D. + 3′-Me-4-DAB
	Experimental† (2)	58	2000	1200	158	31.6	31.6	Put on B.D. 48 hrs before experiment
>160 Days	Control	38	1910	760	165	33.0	23.0	B.D.
	Experimental (204 days)	46	1910	1470	225	45.0	32.7	B.D. + 3′-Me-4-DAB

*Incubated mixture: homogenate 0.5 ml, tris buffer 4.5 ml, whole liver homogenized in 100 ml isotonic saline.
†See footnote 8.

DISCUSSION

From the evidence presented by paper chromatography, it must be concluded that no AMP deaminase or dAMP deaminase is present in detectable amounts in mouse Sarcoma 180, though strong A deaminase and dA deaminase activities can be observed polarographically. In Sarcoma 180 the deamination of AMP and dAMP does not proceed through IMP or dIMP, as in the muscle tissue, but by way of dephosphorylation to the nucleosides, A and dA, and subsequent deamination to I and dI, respectively. The formation of hypoxanthine by both tissues and from both substrates points to further splitting of the nucleosides.

The results also indicate that the enzymatic deaminations of both AMP and dAMP by normal rat liver and Novikoff tumor are due to the action of two enzymes: a phosphatase in the subcellular granules and A deaminase in the supernatant. Deamination is preceded by dephosphorylation of the nucleotides according to the reactions AMP→A→I and dAMP→dA→dI. In the case of AMP the phosphatase (5′-nucleotidase) is present in the microsomal fraction (*2*); in the case of dAMP the phosphatase, a deoxynucleotidase, is present in the mitochondria (*5*). This is confirmed for dAMP by the quantitative agreement between the amount of inorganic phosphate liberated and the amount of substrate deaminated, and for AMP not only by a quantitative agreement between dephosphorylation and deamination in short-time experiments (text-fig. 2), but also by the inhibition of AMP deamination by inorganic phosphate (text-fig. 3). In agreement with these findings are the absence of IMP or dIMP among the reaction products detected by paper chromatography, and the fact that no purified fraction can alone deaminate either AMP or dAMP.

All these findings are evidence against the assumption (*10, 11*) that the enzymatic deamination of AMP is due to the presence of AMP deaminase in rat liver and liver tumors. The failure of Chan *et al.* (*10, 11*) to observe any liberation of inorganic phosphate from homogenates of Novikoff tumor after 15′ incubation with AMP is surprising, especially since whole homogenates, in which the phosphatase (5′-nucleotidase) is abundant in the microsomes, were incubated. Although these authors reflected on the possibility of a combination of enzymes acting in AMP deamination, they neither tested fractions of Novikoff tumor for enzyme activity nor looked for IMP by paper chromatography. On the basis of the experimental evidence, therefore, the hypothesis (*10, 11*) that an increased formation of IMP may facilitate the conversion between adenine and guanine compounds and thus the RNA synthesis in liver tumors is not warranted.

On a cell basis, each of the enzymes involved in AMP and dAMP deamination was lower in Novikoff tumor than in rat liver. In liver carcinogenesis induced by 3′-Me-4-DAB the rate of A deamination was about 100 percent higher 56 days after initiation of carcinogen feeding. Unlike the increased activity of dAMP deaminase, however, the increased

level of A deaminase is not an essential feature of tumors. It is probably one of various manifestations of the adaptive capacity of the liver cell to cope with the azo dye.

Finally, it seems obvious from our experimental data that the enzymatic deamination of AMP cannot be considered of any primary significance for tumor growth, nor has the enzymatic deamination of dAMP this significance for cell proliferation. The rapid deamination of both AMP and dAMP by tumor tissue is not a result of increased activity of AMP deaminase or dAMP deaminase, neither of which exists in the rat liver and liver tumors, but is due solely to the greater cell number per unit of weight in tumor as against corresponding normal tissue. As in tumor respiration (*23*), these conclusions again emphasize the importance of expressing all comparative data in carcinogenesis on a per-cell or unit of DNA basis.

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Mammalian Chromosomes *In Vitro*. XIV. Genotypic Replacement in Cell Populations[1,2]

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SUMMARY

In the 1958 sample, cells of strain L-M (a subline of strain L carried in the Ann Arbor, Mich., laboratory) possessed 2 marker chromosomes, the D and T. The D chromosome was a large, subtelocentric chromosome whose long arm could be subdivided into 4 segments by 3 secondary constrictions, the last constriction being most conspicuous. The T chromosome was probably an isochromosomal derivative of the former. In the 1960 sample of the same strain, those 2 markers disappeared from the stem line. The stem cells contained 2 new marker chromosomes: the E, a chromosome similar in shape to D, but with the long arm having only 3 segments, and the F, a subtelocentric with a very short, knoblike second arm. The long arm of F was identical to that of D in morphological characteristics. New genomes are constantly generated in cell populations *in vitro*. Some of the new genomes, with adaptional advantages or superior growth capacity, may replace the original stem line. Special marker chromosomes may enable investigators to identify the origin of some particular sublines.—J. Nat. Cancer Inst. 26: 1075–1083, 1961.

WHEN NORMAL tissues are subjected to continuous cultivation *in vitro*, the cells often undergo changes, notably in their growth capacity, cell morphology, and chromosome constitution. Sometimes cells from strains of normal origin may become transplantable; in other cases, originally transplantable malignant cells may lose this characteristic after prolonged residence in cell culture.

In established cell strains, genetic polymorphism is a common phenomenon (*1*). From such cell populations, metabolic mutant variants can be selected (*2–7*) when the cells are provided with a suitable environment. Hsu and Kellogg (*8*) applied a mitotic poison (colchicine) to a cell population and recovered sublines that differed from their parental line in chromosomal constitution and growth potentialities.

A recent article (*9*) demonstrated that various genomes in a cell population may have individual metabolic optima, so that the population

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structure may change in a cyclic manner according to the cultural conditions. However, directional changes of population structure may occur when one genome is superior to the other. In most instances in which metabolic or cytological distinctions cannot be easily made, the alteration of population structure can be only inferred. This paper reports a spontaneous shift of population structure as revealed by cytological analysis.

MATERIALS AND METHODS

In this study we used strain L (clone 929) mouse cells (*10*), carried in the Department of Bacteriology, The University of Michigan, and designated by Hsu (*11*) as L-M. This cell line has been maintained continuously for 4 years as monolayer cultures in medium 199 (*12*) plus 0.5 percent Bacto-Peptone (*13, 14*). Before 1959 this medium also contained 100 units of penicillin and 100 μg of streptomycin per ml. Since March, 1959, no antibiotics have been used. Details of the growth of this cell line have been published (*15–18*).

We transplanted cultures at 10-day intervals by scraping a monolayer into fresh medium. A uniform cell suspension was attained by vigorous trituration with a 10 ml pipette. Cells were planted at a concentration of 2 to 3 $\times$ 10^5/ml in medium adjusted to *p*H 7.4 and incubated at 35 $\pm$ 2° C. The medium was renewed on the 6th or 7th day. The mean generation time was approximately 35 hours. At the time of transfer the population was generally between 1.2 and 1.8 $\times$ 10^6 cells/ml.

Prior to 1958 these cells had been maintained at The University of Michigan for 5 years. From 1953 to 1955 the cells were grown in a medium composed of horse serum or 40 percent human ascitic fluid, 20 percent embryo extract, and 40 percent balanced salt solution (BSS). In 1955 the growth medium was changed to medium 199 plus 40 percent horse serum. The horse-serum content was gradually reduced to 5 percent and in 1956 was replaced by 0.5 percent Bacto-Peptone.

In February, 1959, all the cell cultures carried in the Tissue Culture Laboratory of the Department of Bacteriology, The University of Michigan, were checked for the presence of pleuropneumonia-like organisms (PPLO's). Along with most other lines in this laboratory and in keeping with the findings of many other workers, the L strain was found to be contaminated. Subsequent treatment with tetracycline cleared the line of contamination and it has since remained free.

Prior to being tested for the presence of PPLO's, streptomycin (100 μg/ml) and penicillin (100 units/ml) were routinely added to the culture medium. Since March, 1959, all stock lines have been carried without antibiotics.

A culture of L-M was shipped to the Houston laboratory in January, 1958. Only one set of cytological characterizations was made on this cell population after it had been grown for 2 subcultures in Eagle's basal medium containing 5 percent horse serum.

A second shipment from Ann Arbor to Houston was made in January, 1960. These cells were propagated in McCoy's 5a medium (*19*) containing 10 percent calf serum. Three separate samples were taken for analysis during the first 2 months in the Houston laboratory. An additional sample was taken approximately 4 months later. Cytological preparations were made by the conventional colchicine-hypotonic-aceto-orcein squash technique (*1*).

RESULTS

Chromosomes of L Cells

Generally cells of strain L contain from 60 to 75 chromosomes, among which 10 to 20 are biarmed (metacentric and subtelocentric). In a number of sublines minute chromosomes may be common. Most prominent, however, is a long, subtelocentric chromosome with a deep, secondary constriction in the long arm (*7*). This chromosome, known as D, can thus be roughly divided into 3 sections: the short arm, the middle piece, which is approximately twice as long as the short arm, and the end piece located at the distal side of the secondary constriction. The middle piece can be subdivided into 3 sections by 2 less conspicuous secondary constrictions. The first section of the middle piece, adjacent to the centromere, is approximately as long as the short arm. The other 2 segments are of about equal length, and each is approximately half as long as the first. The D chromosome has been found in a number of sublines.

1958 Population of Strain L-M

In the 1958 population of L-M (*9*), the mean total chromosome number per stem cell was 67.36 ± 3.28, that for the metacentrics (M), 13.44 ± 1.86, and that for the subtelocentrics (S), 2.24 ± 0.74. The frequency of the D chromosome per cell was 0.30. However, in a small fraction of cells in which the D chromosome was absent, a very large metacentric was observed (frequency 0.14). This chromosome possessed 1 deep, secondary constriction on each arm. Termed the T chromosome, it was considered to be a derivative of the D element because each arm of the T was morphologically identical with the long arm of the D. It is possible that the D chromosome at first lost its second arm, and later, by misdivision of the centromere, became an isochromosome.

1960 Population of Strain L-M

Several major cytological differences were found between the 1958 and the 1960 populations:

1) The D chromosome was completely missing in the stem cells of the 1960 samples. After examining numerous cells, we encountered this element only occasionally in multiple stem cells (high polyploids).

2) The frequency of the T chromosome was also drastically reduced in the 1960 sample. Among 417 cells in the stemline zone recorded, only 4 (frequency 0.0096) possessed the T element. The T chromosome was also found in multiple stem cells.

3) Minute chromosomes, very frequent in the 1958 population, were rare in most stem cells.

4) Two unique chromosomes were extremely common in cells of the 1960 population: (*a*) A subtelocentric chromosome with a deep secondary constriction in its longer arm, henceforth referred to as the E chromosome, resembled the D chromosome in shape, except for the middle piece. With favorable fixation, this piece could be subdivided by a less conspicuous secondary constriction into 2 segments (3 for the D chromosome). The segment adjacent to the centromere was approximately twice as long as the other (fig. 1). (*b*) A subterminal, long chromosome with a very short, knoblike second arm had a long arm very similar in length and in form to that of the D chromosome. This element will be referred to as the F chromosome (figs. 1 and 2).

Unlike the D and the T chromosomes in the 1958 population, where they did not coexist in a single cell, the E and F chromosomes in the 1960 sample were frequently found to be present together. Table 1 lists the frequency distribution of these 2 elements among 417 cells. Cells with 1 E and 1 F were most common.

TABLE 1.—Frequency distribution of the E and F chromosomes among stem cells of strain L-M, 1960 sample

F \ E	0	1	2	3	Number of cells
0	28	102	54	3	187
1	15	182	21	1	219
2	1	7	2	1	11
Number of cells	44	291	77	5	417

To ascertain that the E and F chromosomes were not induced aberrations after a change of medium in the Houston laboratory, two more shipments from Ann Arbor to Houston were made in August, 1960. In addition to the L-M stocks grown in 199-peptone medium, another subline adapted to Eagle's medium (double strength) without peptone or serum was included. Both sublines were grown in their respective media shipped from Michigan to the Houston laboratory and were fixed as soon as the cultures began to proliferate. Both chromosomes were found in these lines. Thus the genotypic change occurred not only in the Michigan laboratory, but prior to the adaptation of the line from 199-peptone medium to peptone-free medium. An additional proof that these chromosomes originated in Ann Arbor was found in the laboratory of Dr. C. C. Randall, Department of Microbiology, The University of Mississippi Medical School, Jackson, Mississippi, when one of us visited that institution. The L strain carried in that laboratory clearly showed

both the E and F chromosomes. The strain was originally supplied by the Ann Arbor laboratory and had no contact whatever with the Houston laboratory.

DISCUSSION

Spontaneous alterations of population structure have been observed with animal cells. In transplantable tumors, such as the Krebs-2 and the Ehrlich carcinomas of the mouse, Levan (*20*) noted a consistent decrease of chromosome number over a period of 2 years. In strain L-P55, a spare stock lost 4 chromosomes in 2 years (*4*). In this particular instance the changed population utilized galactose efficiently, whereas the parental population did not. Recently, Ohno and associates (*21*) reported a shift of the stem line in a mouse tumor from all telocentrics to one with a metacentric. The factors responsible for such changes are not clear, but it is reasonable to think that the new genomes replacing the old are more vigorous under the given conditions.

The present case is another example of genotypic replacement in cell populations. In 2 years, the strain lost approximately 3 chromosomes per cell (67 in 1958 vs. 64 in 1960), and lost both its D and T chromosomes. Instead, the 1960 population possessed new genomes with new marker chromosomes, the E and F. It is almost beyond doubt from morphological criteria and the concomitant loss of the D that these 2 new chromosomes, like the T chromosome, were also the derivatives of the D—the E by deletion of a segment in the middle piece and the F by loss of most of the short arm.

Since the D chromosome can be found in many sublines of strain L, it may have had a long history and thus is likely advantageous under most cultural conditions. The structural changes which created the T chromosome must also be beneficial for growth under the conditions used for maintenance of this strain. Whether it emerged as a result of growth in protein-free medium remains a question. During 1958 and 1959, more structural changes took place in strain L-M. It appears that cells containing the new elements, E and F, were so vigorous that they even replaced the original genomes.

The cells have become better adapted to growth in simple medium during this interval. While the initial adaptation of strain L-M (1959) to growth in chemically defined medium required a somewhat prolonged period, it is possible now (1960) to convert cells rather quickly from 199-peptone medium to double-Eagle's. One particular event that took place between the 1958 study and those made in 1960 is also worth mentioning. This was the clearing of PPLO's from the cell line by treatment with tetracycline early in 1959. The treatment required the use of relatively large amounts (100 μg/ml) of tetracycline continuously for approximately 3 weeks. This concentration of tetracycline approaches the toxic level for strain L cells growing in a protein-free medium. It is

possible that tetracycline induced chromosome breakages or acted as a selection factor.

Special marker chromosomes usually enable cytologists to identify cell strains not only to the species level, but occasionally to a particular subline. The identification of strain L-M in The University of Mississippi, without the previous knowledge of the origin of the culture, is a dramatic illustration of the usefulness of karyological examination when the sources of cultures or cellular contamination are traced as a routine procedure for tissue-culture laboratories.

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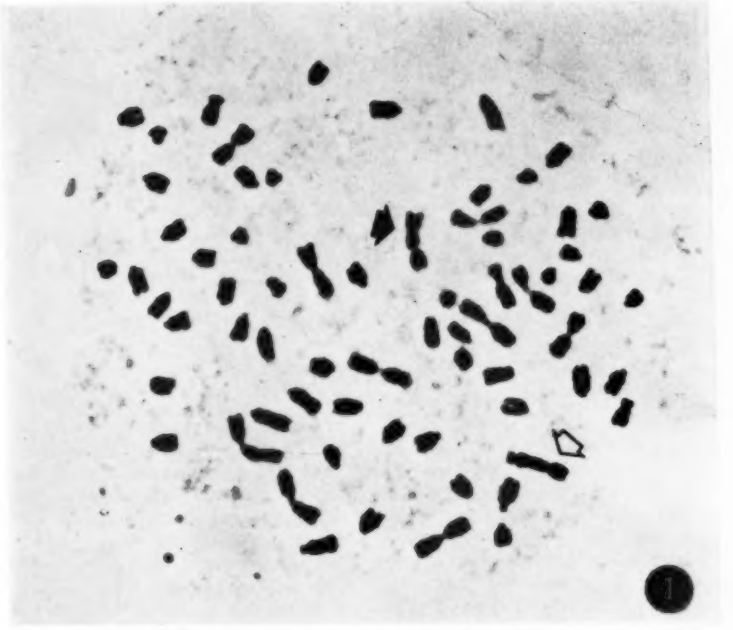
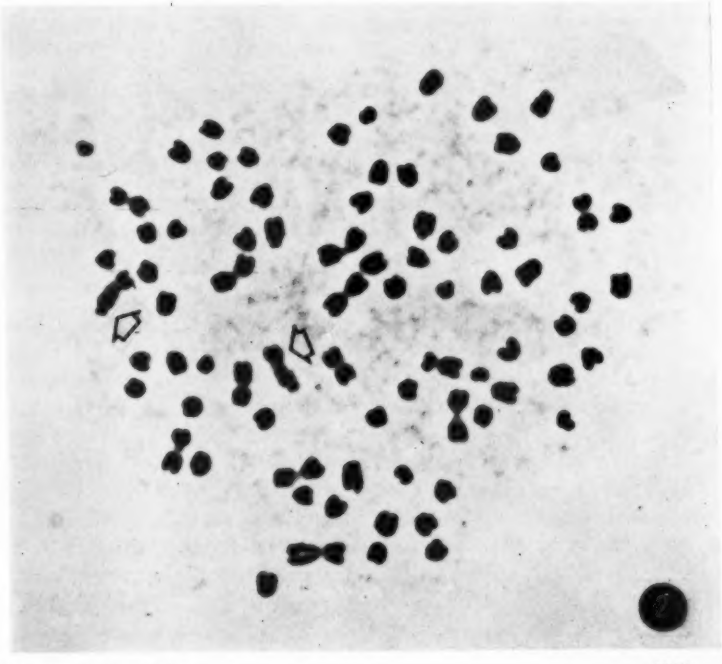
FIGURE 1.—Cell from 1960 sample of strain L-M; 67 chromosomes. *Solid arrow*, E chromosome; *open arrow*, F chromosome.

FIGURE 2.—Cell from 1960 sample of strain L-M; 77 chromosomes. *Open arrow*, F chromosome.





Results of a French Survey on the Role of Tobacco, Particularly Inhalation, in Different Cancer Sites [1]

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SUMMARY

The results of a retrospective study of 3,937 cancer patients are presented, with controls matched by age and interviewer. A list of sites associated with smoking is established, including the lip, tongue, oral cavity, oropharynx, hypopharynx, larynx, lung, esophagus, and bladder. By considering the different criteria of consumption, we were able to separate these sites into two groups: (*a*) In cancer of the lip, oral cavity (excluding the tongue), oropharynx, hypopharynx, and esophagus, neither inhalation nor preference for cigarettes seems to be significant. (*b*) Inhalation and preference for cigarettes are significant for cancer of lung, larynx, and probably bladder. In the sites listed in (*b*), inhalation was an important factor, independent of the amount smoked or the method of smoking. The particular significance of the cigarette is less definite; it depends, in part, but in part only, on inhalation, which is associated with this type of smoking. To give a complete explanation of the significance of cigarettes, we have included other considerations: Smokers of the cigarette-only, pipe-only, and pipe-and-cigarette categories differ in certain respects and perhaps in their manner of smoking in general. The effect of inhalation appears, in this study, to be specific. Thus it is significant in some sites of cancer but not in others, and the study of a group of noncancerous patients brings out the relationship between inhalation and certain diseases.—J. Nat. Cancer Inst. 26: 1085–1108, 1961.

THE ROLE of tobacco in different cancer sites in man has already been discussed in several well-known reports from various countries. Most studies agree and certain points have been clearly established, yet others remain controversial, such as an exact list of affected sites and various aspects of the effect of smoking habits—particularly inhaling. Our purpose is to show the results of a French survey in this respect.

The preliminary results of this study, which was started in 1954, have been published in two papers: a detailed study related to lung cancer (*1*) and a shorter study on all cancer sites (*2*). Thus, we are now able to give detailed results for all cancer sites.

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DEFINITION OF THE SURVEY

This retrospective study concerns males admitted to hospitals in Paris and a few large provincial cities. A team of specialized interviewers sorted and interviewed the largest proportion possible, if not all the cancer patients in these hospitals. During the first 2 years our chief research was on cancer of the lung, which accounts for its predominance in this study; later research extended to all sites.

For each cancer patient interviewed there were 2 types of controls: a patient with some other disease than cancer, who was interviewed in a general medical ward, and a healthy individual, who had been admitted to the hospital because of a work or traffic accident. Each cancer patient and his 2 controls were of the same 5-year age group and were interviewed by the same person during the same period at the same group of hospitals.

In this way 3,937 cancer patients and 3,614 controls were interviewed. The control group was not twice the number of the cancer group, because each pair of controls served for several *different* cancer sites (an average of about 2 sites, according to the figures given).

The interview (*3*) lasted approximately 20 minutes. It included questions on the area of residence, family status, occupation, past medical history, feeding habits, etc. The section on tobacco was particularly detailed and concerned smoking at the various periods of the subject's life, in order to study the following points:

Method of smoking.—We distinguished 3 categories of smokers, according to whether they smoked cigarettes, pipe, or both ("mixed" smokers). Cigar smoking was negligible and was therefore ignored (less than 1 person out of 300 smoked more than 1 cigar a day). A patient was considered a cigarette smoker if he had smoked an average of at least 1 cigarette a day during the 10 years preceding his disease (or the date of the interview in the controls), and a pipe smoker if he had smoked at least 1 g of tobacco a day during this period. The others were nonsmokers.

Amount of smoking.—This is the average quantity smoked per day during the reference period. The quantity of cigarettes smoked will be expressed in number of cigarettes per day, and the quantity of pipe smoking in an equivalent number of cigarettes per day (ecd) on the basis of 1 g of pipe tobacco = 1 cigarette. The ecd will also express the total consumption by smokers, other then those in the "cigarrette-only" category.

Smoking habits.—We have taken special interest in whether the subjects inhaled the smoke. For the first 2 years of the survey, we asked the smokers if they were in the habit of rejecting the smoke *immediately* (through the mouth or nose), or of inhaling it deeply and rejecting it *afterward* (through the mouth, nose, or both). From the 3d year on, we asked the subjects only if they were in the habit of "swallowing" the smoke—this word is currently used in France to designate inhalation. We observed no difference between the results of this period and those of the 2 earlier years.

The habit of rolling cigarettes will not be discussed. An earlier paper (*2*) described some interesting points, but they do not seem valid when sampling factors are taken into account.

FIRST ANALYSIS AND CHOICE OF METHODS. CONTROLS

Our first concern was to compare smoking habits of the 2 control groups: the *ill subjects* (noncancerous) and the *healthy subjects* (accident cases). Table 1 shows that these groups are similar in both proportion of smokers and method of smoking, but the ill subjects smoke a slightly greater quantity and they inhale more often; this latter difference is highly significant and important enough for us to have sought an explanation for it from the outset.

The ill subjects had entered hospitals for various diseases, some of which could be related to the use of tobacco in general and to the inhalation of smoke in particular. Classifying these patients according to diagnosis on admission, we observed (*see* Appendix 1) a relationship between inhalation and various diseases, notably arteriosclerosis, some lung disorders, and various symptomatic disturbances (digestive, cardiovascular, etc.). The proportion of ill controls hospitalized for these diseases accounts for the difference between the two control groups.

This observation led us to draw two conclusions: 1) Inhalation might be more important in the etiology of certain diseases, *i.e.*, arteriosclerosis, than the amount smoked. A more detailed examination of this relationship is beyond the scope of the present paper and will be the object of a later study. 2) The control group of *ill subjects* is not a valid control group; the group of *healthy subjects*, however, appears to be entirely acceptable, so it will henceforth be adopted as the control group.

TABLE 1.—Comparison between the 2 control groups with regard to the various criteria of consumption*

	Number of cases	Smokers (percent)	Method of smoking: percent smoking only cigarettes		Total amount smoked by smokers (ecd) †	Percent of subjects who inhaled cigarette smoke
			Among smokers	Among cigarette smokers		
Ill controls	1, 807	82	86	90	15. 9	49
Healthy controls	1, 807	81	86	90	15. 3	41
Significance $P =$					0. 05	0. 001

*Smokers include cigarette-only, cigarette-and-pipe, and pipe-only smokers. Cigarette smokers include cigarette-only and cigarette-and-pipe smokers.

†Equivalent number of cigarettes per day.

Sampling Factors

According to our earlier studies (*3*), the controls differ from cancer patients only in regard to 3 sampling factors: area of residence (urban or rural), family situation, and occupation.

Table 2 indicates these differences in sampling: There is a greater proportion of urban population among the controls than among the cancer patients, who have come a long distance because of the serious nature of their disease; controls are more frequently bachelors, widowers, or divorcés—a family situation favoring hospitalization but of little consequence in a serious disease; finally, the distribution of occupations differs somewhat from one group to the other.

TABLE 2.—Differences of sampling between controls and cancer patients

	Number of cases	Area of residence: city/suburbs/ rural area (%)	Family status: bachelors, widowers, or divorcés (%)	Occupation: professionals, senior staff members/shopkeepers, craftsmen, employees/ manual workers/ farmers (%)
Controls	1, 807	53/39/8	33	4/32/63/1
Cancer patients	3, 937	48/29/23	22	4/36/55/5
Significance		Highly significant ($\chi^2 = 181$ with 2 d.f.)	Highly significant ($\chi^2 = 79$ with 1 d.f.)	Highly significant ($\chi^2 = 63$ with 2 d.f.)

These differences are significant because of the large number of subjects, but they remain small; only the area of residence differs notably.

Differences of sampling enter into account only insofar as they affect smoking. Table 3 indicates these relationships: The most important

TABLE 3.—Smoking and sampling factors (control group, 1,807 subjects)*

	Percent smokers	Method of smoking: smoking only cigarettes (%)		Total amount smoked by smokers (ecd)†	Percent of subjects who inhale cigarette smoke
		Among smokers	Among cigarette smokers		
Area of residence					
Rural area	77	83	88	15. 3	39
Suburbs	81	87	90	15. 0	40
City	81	85	90	15. 5	42
Significance P =					
Family status					
Bachelors, widowers, divorcés	79	87	91	15. 7	46
Married	82	85	89	15. 0	38
Significance P =					0. 01
Occupation					
Professionals, senior staff members	75	78	84	14. 8	33
Shopkeepers, craftsmen, employees	77	85	90	15. 5	40
Manual workers	83	86	90	15. 2	42
Farmers	68	100	100	14. 3	27
Significance P =	0. 01				

*See footnote, table 1.

†Equivalent number of cigarettes per day.

factor, area of residence, is not associated with any of the criteria of consumption. The two other factors do show an association: Family status has an influence on inhalation, and occupation on the proportion of smokers. These associations are slight, however, and since they bear on sampling factors which differ little between cancer cases and controls, their effects are minimal. Under these conditions there is no fear of a sampling bias. To be thorough, however, all calculations made at first with the raw data were later repeated, with the three sampling factors considered. Results were never affected in any way by these corrections. We have used the raw data for our tables.

Table 4 shows the relation of tobacco consumption to age. This is not useful for statistical analysis, since cancer patients and control subjects are matched with regard to age, but it contributes interesting information. Older subjects are less frequently smokers, consume less than the young, smoke the pipe more often, and are much less inclined to inhale cigarette smoke. This last and extremely significant trend agrees with the observations of Lickint (*4*) and Hammond (*5*). These various trends do not necessarily reveal an effect of age. They may indicate that in the last decades the preference for cigarettes and the habit of inhaling have increased.

TABLE 4.—Tobacco consumption and age (control group, 1,807 subjects)*

	Number of cases	Percent smokers	Method of smoking: percent smoking only cigarettes		Total amount smoked by smokers (ecd)†	Percent of subjects who inhale cigarette smoke
			Among smokers	Among cigarette smokers		
Age −50	407	88	88	92	16. 8	51
50–59	808	82	88	91	15. 4	43
60+	592	74	80	86	13. 9	29
Significance		Highly significant ($\chi^2=32$ with 2 d.f.)	0. 001	0. 01	Highly significant ($F=9.9$ with d.f. 2 and ∞)	Highly significant ($\chi^2=40$ with 2 d.f.)

*See footnote, table 1.
†Equivalent number of cigarettes per day.

Relationship Between Criteria of Consumption

These different criteria (amount smoked, method of smoking, and tendency to inhale) are very much interrelated, as shown in table 5 (A and B).

1) *Relationship between amount smoked and method of smoking.*—The "mixed" smoker is the most heavy (21.3 ecd), whereas the cigarette-only or pipe-only smokers consume 14.5 ecd and 16.3 ecd, respectively; these differences are highly significant.

2) *Relationship between the amount smoked and inhalation.*—Heavy cigarette smokers are more inclined to inhale cigarette smoke. Thus,

TABLE 5.—Relationship between the criteria of consumption

A.—Smokers*

	Total amount smoked (ecd)†	Method of smoking: percent of cigarette-only smokers
Total amount smoked (ecd)		
1–9		88
10–19		85
20–29		89+++
30+		64
Method of smoking		
Cigarette	14.5	
Cigarette and pipe	21.3+++	
Pipe	16.3	

B.—Cigarette smokers*

	Total amount smoked (ecd)†	Number of cigarettes per day	Method of smoking: percent cigarette-only smokers	Percent of subjects who inhale cigarette smoke
Total amount smoked (ecd)				
1–9		4.9	94	26
10–19		12.5	90	44
20–29		20.1+++	91+++	46+++
30+		32.1	71	58
Number of cigarettes per day				
1–9	6.2		86	25
10–19	13.4		91	45
20–29	21.0+++		93++	47+++
30+	36.6		85	64
Method of smoking				
Cigarette	14.5	14.5		44
Cigarette and pipe	21.3+++	13.6		20+++
Subjects who inhale cigarette smoke				
No	13.9	12.9	86	
Yes	17.1+++	16.6+++	95+++	

*Smokers include cigarette-only, cigarette-and-pipe, and pipe-only smokers. Cigarette smokers include cigarette-only and cigarette and pipe smokers. Significance is +, ++, +++ for $P = 0.05$, or 0.01, or 0.001 and below.
†Equivalent number of cigarettes per day.

among cigarette smokers, one notes the following significant progression ($\chi^2 = 72$ with 3 d.f.):

Amount of smoking (ecd)	1 to 9	10 to 19	20 to 29	30+
Percent of subjects inhaling	25	45	47	64

This relationship has already been pointed out (*1*, *4*, *5*).

Our proportion of subjects inhaling, chiefly among heavy smokers, is clearly lower than in the States (*5*). It is not possible to say if this difference is a result of the question asked or reflects a more basic difference between French and American habits. The second hypothesis seems

more likely to us since we have obtained similar results when asking this question in two different ways according to the period of the survey.

3) *Relationship between inhalation and method of smoking.*—"Mixed" smokers are less inclined to inhale *cigarette* smoke than are smokers of cigarettes only: 20 times out of 100 against 44, a very highly significant difference ($\chi^2 = 30$ with 1 d.f.). We have already given our interpretation of this fact (*1*) by indicating that pipe smokers very rarely inhale and must extend this habit to cigarettes when they smoke both. Hammond (*5*) has observed the same fact in cigarette and cigar smokers. The proper role of each criterion of consumption can be studied only by taking the other criteria into consideration.

Method of Analysis

To allow for both the sampling factors and the interrelation of criteria of consumption, we have had to deal with such problems as comparison of proportions p and p' of subjects inhaling, area of residence being similar, and amount smoked being equal; in other words, level of variables x, y, etc., being equal.

For *one* variable x we have considered various classes x_i (*i.e.*, city, suburbs, rural area) and compared the proportions p and p': *a*) for each class x_i (comparison of each difference $p_i - p'_i$ to 0); *b*) for the whole, reducing cancer and control groups to a same distribution of x_i. This adjustment amounts to comparing to 0 a combination $\Sigma a_i\ (p_i - p'_i)$, in which the weights a_i are adequately chosen. For this choice we have adopted Cochran's method (*6*) based on the reasonable hypothesis that differences $(p_i - p'_i)$ are about equal on the probit scale.

The previous examples dealt with a qualitative criterion, *i.e.*, subject does or does not inhale, and led to comparisons of percentages. With quantitative criteria, *e.g.*, number of cigarettes a day, averages were compared through an analogous process of adjustment, which formed combinations with suitable weights. Results will sometimes be expressed by the use of the relative risk defined by Cornfield (*7*).

RESULTS

Table 6 shows the raw data for cancer at various sites in cancer patients and their corresponding controls. Cancer patients are compared with their matched controls rather than with all controls. This was deemed desirable since, as already described, matching was not only on age, but also on interviewer, date of interview, and hospital group. In the subsequent tables minor differences in numbers of individuals may result because the necessary data are missing for some cases.

Cancers Associated With Smoking

In table 6 the column relative to the proportion of smokers brings out an association with lips, tongue, oral cavity (excluding the tongue),

TABLE 6.—Comparison of cancer at different sites in cancer patients and controls*

International Classification number (sixth edition)	Site	Number of cases	Percent smokers	Total amount smoked by smokers (ecd)†	Percent cigarette-only smokers: Among smokers	Percent cigarette-only smokers: Among cigarette smokers	Percent of subjects inhaling cigarette smoke among cigarette smokers
140	Lips	Cancer 49	92 ++	16.6 +	91	95	26
		Controls 49	71	12.1	83	88	30
141	Tongue	Cancer 164	95 +++	16.0 +	86	92	53 +
		Controls 164	81	13.7	83	90	38
143, 144	Oral cavity (other sites)	Cancer 144	97 ++	18.1 ++	86	92	48
		Controls 144	88	14.6	88	90	46
145	Oral mesopharynx	Cancer 141	99 +++	19.6 ++	86	90	47
		Controls 141	77	15.4	92	93	45
160	Nasal cavities	Cancer 53	72	16.4	95	97	54
		Controls 53	81	13.5	84	90	45
147	Hypopharynx	Cancer 206	96 ++	19.1 +++	82	86	48
		Controls 206	88	15.1	83	87	42
161	Larynx	Cancer 249	98 +++	19.7 +++	91++	93	60 +++
		Controls 249	82	15.6	83	90	40
162	Lung	Cancer 1,159	96 +++	20.1 +++	93+++	94+++	58 +++
		Controls 1,159	80	15.6	84	89	41
150	Esophagus	Cancer 362	97 +++	16.8	82	87	39
		Controls 362	83	16.0	85	90	38
151	Stomach	Cancer 263	84	14.6	89	92	37
		Controls 263	83	15.3	83	87	34
152, 153, 154	Intestine and rectum	Cancer 355	84	14.2	84	89	36

152, 153, 154	Intestine and rectum	Cancer	355	84		14. 2		84	89	36	
		Controls	355	83		14. 7		84	88	36	
155, 157, 158	Other digestive localizations	Cancer	61	89		14. 7		89	96	52	
		Controls	61	84		16. 4		86	90	45	
191 (1 to 3)	Skin (face)	Cancer	89	83		15. 6		90	93	37	
		Controls	89	80		14. 0		86	90	34	
191 (4 to 9)	Skin (face excepted)	Cancer	65	74		17. 8		88	89	47	
		Controls	65	86		15. 8		86	92	41	
180	Kidney	Cancer	69	78		15. 0		89	89	44	
		Controls	69	83		15. 0		86	86	46	
181	Bladder	Cancer	214	89	++	16. 9	++	93	94	54	++
		Controls	214	80		14. 4		88	91	37	
177	Prostate	Cancer	139	79		13. 6		90+	90	36	
		Controls	139	73		13. 8		77	82	34	
178	Testis	Cancer	37	76		14. 6		86	86	67	
		Controls	37	89		13. 6		91	91	45	
179	Penis	Cancer	32	84		14. 9		89	92	23	
		Controls	32	84		16. 7		85	88	19	
192 to 197	Nervous system Endocrine glands Various sites	Cancer	86	76		15. 2		89	92	44	
		Controls	86	86		14. 6		86	90	34	

See footnote, table 5.

† Equivalent number of cigarettes per day.

oropharynx (oral mesopharynx), hypopharynx, larynx, lung, esophagus, and bladder. These associations are all highly significant: The probability of chance is in all cases inferior to 1 per 100 (to 1 per million for cancers of the oropharynx, larynx, lung, and esophagus).

Examination of *other sites* is instructive: It appears that the lack of significance cannot be systematically attributed to the small number of subjects, since in the cases of gastric cancer (263) and cancer of the intestine and rectum (355), cancer patients and controls are very comparable. This similarity is an important argument in confirming both the validity of the controls and the specificity of association with certain sites.

Other Criteria of Consumption

The columns of table 6 concerning amount smoked, method of smoking, and inhalation bring out the following points:

1) These results agree remarkably with those concerning the proportion of smokers: (*a*) The sites *presenting no association with smoking* do not, as a whole, present any association with any single criterion of consumption; the only exception to this rule is the association between cancer of the prostate and cigarette smoking. The level of significance, however, is $P = 0.02$, and the great number of comparisons caused a critical attitude toward weak significance. (*b*) However, for *sites associated with smoking*, cancer cases differ from controls in various criteria, and always in the total amount smoked, which is significantly higher. The only exception is cancer of the esophagus. Although this is closely related to the use of tobacco, the amount smoked by cancer patients and controls is closely comparable. This fact calls for confirmation and interpretation.

2) There is a sharp separation into two groups: (*a*) In cancers of the lung, larynx, and bladder, the smokers show a greater tendency than their controls to inhale cigarette smoke; this association is very significant ($P = 0.01$ for the bladder, 1/10,000 for the larynx, and 1/1 billion for the lung). They more often smoke cigarettes, which is significant in cancers of the lung and the larynx and quite large, but not significant in cancers of the bladder. (*b*) These differences, however, do not exist in cancers of the oral cavity (tongue excluded), oropharynx, hypopharynx, and esophagus. Cancer of the tongue, which for anatomical reasons is in this group, does present a significant association with inhalation. But this association is significant only to 0.02. Since the cigarette does not seem to be a factor, this significance should be accepted with some reserve.

The foregoing results are based on the data of table 6. These are *raw* data but, as we have already pointed out, correction for sampling factors does not make a difference.

Detailed Analysis

The importance of criteria of consumption and their interrelation remain to be examined in cancers of the lung, larynx, and bladder. A discussion of the principal results of this analysis follows.

1) *Amount smoked and method of smoking.*—The heavier smokers are as a rule the "mixed" smokers; however, patients with cancer of the lung, larynx, and bladder are heavy smokers and have special preference for cigarettes only; neither of these propositions can result from the other. Their related roles appear in tables 7, 8, and 9.

TABLE 7.—Cancer of the lung: amount smoked and method of smoking by cancer patients and controls

A.—Total amount smoked in relation to method of smoking (smokers)*

Method of smoking	Number of cases		Amount smoked (ecd)†		
			Cigarettes	Pipe	Total
Cigarette only	Cancer	1,041	19.8+++		19.8+++
	Controls	779	14.7		14.7
Cigarette and pipe	Cancer	64	19.3++	6.5 −25.8	
	Controls	97	14.0	9.1 −23.1	
Pipe only	Cancer	9		14.2	14.2
	Controls	54		15.4	15.4

B.—Method of smoking in relation to total amount smoked (smokers)*

Total amount smoked (ecd)†	Number of cases		Percent of cigarette-only smokers		
			Among smokers	Among smokers who do not inhale ‡	Among smokers who inhale
1–9	Cancer	114	96++	93	98
	Controls	234	86	83	95
10–19	Cancer	345	94+++	92+++	97
	Controls	332	84	77	97
20–29	Cancer	458	94++	92+	99
	Controls	282	89	85	97
30+	Cancer	197	89+++	81+++	97+++
	Controls	82	57	36	80
Adjustment for total amount smoked			+++	+++	++

C.—Method of smoking in relation to number of cigarettes smoked (cigarette smokers)*

Number of cigarettes per day	Number of cases		Percent of subjects who, in addition, smoke a pipe	When the subject, in addition, smokes a pipe, the risk is multiplied by:
1–9	Cancer	119	8	0.5
	Controls	236	14	
10–19	Cancer	343	5−	0.5
	Controls	311	10	
20–29	Cancer	457	5	0.7
	Controls	271	7	
30+	Cancer	187	6−−	0.3
	Controls	58	19	
Adjustment for number of cigarettes per day			−−−	

See footnotes at end of table.

TABLE 7.—Cancer of the lung: amount smoked and method of smoking by cancer patients and controls—Continued

D.—Method of smoking in relation to number of cigarettes smoked (cigarette smokers)*

Number of cigarettes per day		Percent of subjects who, in addition, smoke a pipe		
		Smokers who do not inhale		Smokers who inhale
1–9	Cancer	13	Cancer	5
	Controls	17	Controls	8
10–19	Cancer	7—	Cancer	3
	Controls	15	Controls	4
20–29	Cancer	8	Cancer	4
	Controls	10	Controls	4
30+	Cancer	12—	Cancer	3—
	Controls	32	Controls	13
Adjustment for number of cigarettes per day		——		

*Smokers include cigarette-only, cigarette-and-pipe, and pipe-only smokers. Cigarette smokers include cigarette-only and cigarette-and-pipe smokers. Significance is noted +, ++, +++, for P=0.05, 0.01, or 0.001 and below. Significance, noted −, −−, or −−−, underlines the fact that the level among cancer cases is *lower* than among controls.

†Equivalent number of cigarettes per day.

‡Cigarette or pipe smoke is taken into account for cigarette-only or pipe-only smokers. *Both* are taken into account for mixed smokers.

TABLE 8.—Cancer of the larynx: amount smoked and method of smoking by cancer patients and controls

A.—Total amount smoked in relation to method of smoking (smokers)*

Method of smoking	Number of cases		Amount smoked (ecd)†		
			Cigarettes	Pipe	Total
Cigarette only	Cancer	219	19. 5+++		19. 5+++
	Controls	168	14. 6		14. 6
Cigarette and pipe	Cancer	17	15. 8	6. 2	22. 0
	Controls	18	16. 3	8. 7	25. 0
Pipe only	Cancer	5		18. 8	18. 8
	Controls	17		14. 6	14. 6

B.—Method of smoking in relation to total amount smoked (smokers)*

Total amount smoked (ecd)†	Number of cases		Percent of cigarette-only smokers
1–9	Cancer	39	90
	Controls	55	84
10–19	Cancer	68	91
	Controls	73	85
20–29	Cancer	85	93
	Controls	56	86
30+	Cancer	49	88+
	Controls	19	63
Adjustment for total amount smoked			++

*Smokers include cigarette-only, cigarette-and-pipe, and pipe smokers. Cigarette smokers include cigarette-only and cigarette-and-pipe smokers. Significance is noted +, ++, +++, for P=0.05, or 0.01, or 0.001 and below.

†Equivalent number of cigarettes per day.

TABLE 9.—Cancer of the bladder: amount smoked and method of smoking by cancer patients and controls

A.—Total amount smoked in relation to method of smoking (smokers)*

Method of smoking	Number of cases		Amount smoked (ecd)†		
			Cigarettes	Pipe	Total
Cigarette only	Cancer	178	16. 5++		16. 5++
	Controls	149	14. 1		14. 1
Cigarette and pipe	Cancer	10	19. 1	7. 3	26. 4
	Controls	14	9. 4	7. 2	16. 6
Pipe only	Cancer	2			
	Controls	7			

B.—Method of smoking in relation to total amount smoked (smokers)*

Total amount smoked (ecd)†	Number of cases		Percent of cigarette-only smokers
1–9	Cancer	33	94
	Controls	44	91
10–19	Cancer	71	97+
	Controls	68	85
20–29	Cancer	69	91
	Controls	49	90
30+	Cancer	17	88
	Controls	9	78
Adjustment for total amount smoked			+

*See footnote, table 8.
†Equivalent number of cigarettes per day.

Table 7 gives the findings for lung cancer:

(*a*) According to table 7A, the cancer patients smoke more cigarettes than the controls, regardless of the method of smoking (cigarette-only or cigarette and pipe). Here the cigarette alone is involved; cancer subjects and controls smoke comparable amounts among pipe-only smokers and one observes in the pipe-cigarette category that pipe smoking is *lighter* among the cancer subjects, whose consumption of cigarettes is definitely greater.

(*b*) Yet cancer subjects (table 7B) smoke only cigarettes more often than the controls at an equal level of total consumption do. This remains true for two categories of subjects: those who do not inhale the smoke (of cigarette or pipe) and those who do. The difference is particularly evident among the subjects who do not inhale; thus *the cigarette plays a distinct part, at a given level of total consumption* and *in the absence of inhalation.* Certain peculiarities of the cigarette have been taken into consideration (paper, temperature of combustion, etc.), but these alone cannot account for the role of the cigarette. This is shown by table 7C, where cigarette-smoking cancer patients and controls are compared—consumption of cigarettes being equal. One obtains the apparently paradoxical result, already noted in our earlier publication (*1*), that *out of two subjects smoking the same number of cigarettes a day, the one who, in addition, smokes a pipe incurs only half the risk of lung cancer.*

Here the peculiar properties of the cigarette cannot be considered, since both subjects smoke the same amount. Smoking habits offer a more satisfactory explanation: For instance, we have seen that smokers of the cigarette-only category are more inclined to inhale than are "mixed" smokers. But this is only a partial explanation, for, even if we take inhalation into account, the phenomenon remains (table 7D); it is perhaps less evident, but is still significant, particularly among subjects who do not inhale.

Our interpretation—amplified later—is that the cigarette-only smokers and the "mixed" smokers differ in general smoking habits, inhalation being but one aspect of this difference.

(*c*) Tables 8 and 9 include cancers of the larynx and bladder. Here also the number of cigarettes smoked appears to have a proper significance, as does the method of smoking.

2) *Amount smoked and inhalation.*—In the study of this association we deal with cigarette smoking only. The study of cigarette-pipe smokers yields similar results, but they are less conclusive because of the smaller number of cases. Table 10 gives results for lung cancer.

According to table 10A, the amount smoked plays a distinct part whether subjects inhale or not. The effect seems more marked when they do not inhale.

Table 10B shows that *at an equal level of smoking* cancer patients have a greater tendency to inhale. This peculiarity results not merely from the fact that they are heavy smokers; it has an independent significance, which is all the less apparent when consumption is greater, and it finally disappears completely among heavy smokers.

Both the amount smoked and inhalation have independent significance, and they can compensate each other: Cancer subjects who smoke little inhale much more often than is normal for light smokers (50 times out of 100, instead of 29), and cancer subjects who do not inhale are much heavier smokers than is normal for these subjects (quantity smoked 50% greater).

These relative roles are shown in table 10C, which gives relative risks. The role of consumption reads across; it is less important among subjects who inhale than among those who do not. The role of inhalation reads downward; risk is increased by 2.5 for light smokers; then the effect diminishes as consumption increases.

Tables 11 and 12 show similar data for cancer of the larynx and bladder. For these cancers, each of the two factors, amount smoked and inhalation, maintains a distinct significance, the other factor remaining constant.

3) *Method of smoking and inhalation.*—Here we distinguish two categories of smokers: *a*) those who inhale neither cigarette nor pipe smoke, according to their method of smoking; and *b*) those who inhale either cigarette or pipe smoke or both, according to whether they are of the cigarette-only, pipe-only, or "mixed" smoker category. We do not take into account the mixed smokers who inhale the smoke in only one method of smoking.

TABLE 10.—Cancer of the lung: amount smoked and inhalation (cigarette-only smokers)*

A.—Amount smoked in relation to inhalation *

Inhalation	Number of cases		Number of cigarettes per day
No	Cancer	424	19.5+++
	Controls	440	13.2
Yes	Cancer	615	20.0+++
	Controls	334	16.6
Adjustment for inhalation			+++

B.—Inhalation in relation to number of cigarettes per day *

Number of cigarettes per day	Number of cases		Percent of smokers who do inhale
1–9	Cancer	109	50+++
	Controls	201	29
10–19	Cancer	324	59++
	Controls	278	46
20–29	Cancer	432	61+++
	Controls	249	46
30+	Cancer	174	60
	Controls	46	72
Adjustment for number of cigarettes per day			+++

C.—Relative risks

Inhalation	Number of cigarettes per day				Significance *
	1–9	10–19	20–29	30+	
No	1	2.3	3.3	14.0	+++
Yes	2.5	4.0	6.1	8.4	+++
Significance	+++	+++	+++		

* Significance is noted +, ++, +++, for $P = 0.05, 0.01, 0.001$, and below.

Table 13, relative to lung cancer, shows that the preference for cigarettes only and the tendency to inhale are both individually significant. In cancer of the larynx and of the bladder (tables 14 and 15) we find that inhalation is individually significant, but, in regard to the preference for cigarettes only, its part is barely noticeable (larynx) or nil (bladder).

4) *Inhalation and previous diseases.*—At the beginning of this study as well as in Appendix 1, we have drawn attention to the marked trend toward inhalation among subjects who have in the past suffered from diseases such as arteriosclerosis and bronchitis. It is interesting to compare the cancer patients with the controls. Details of this study are given in Appendix 2 under lung cancer. In summary its principal points are: (*a*) A study of the past medical history of cancer patients confirms the

TABLE 11.—Cancer of the larynx: amount smoked and inhalation (cigarette-only smokers)*

A.—Amount smoked in relation to inhalation*

Inhalation	Number of cases		Number of cigarettes per day
No	Cancer	85	17.2++
	Controls	96	12.4
Yes	Cancer	133	20.8+
	Controls	72	17.6
Adjustment for inhalation			+++

B.—Inhalation in relation to number of cigarettes per day*

Number of cigarettes per day	Number of cases		Percent of smokers who inhale	
1–9	Cancer	35	37	
	Controls	46	20	
10–19	Cancer	62	55	
	Controls	62	50	
20–29	Cancer	79	73	
	Controls	48	46	++
30+	Cancer	42	67	
	Controls	12	83	
Adjustment for number of cigarettes per day				++

*See footnote, table 10.

TABLE 12.—Cancer of the bladder: amount smoked and inhalation (cigarette-only smokers)*

A.—Amount smoked in relation to inhalation*

Inhalation	Number of cases		Number of cigarettes per day
No	Cancer	78	14.1
	Controls	90	12.7
Yes	Cancer	98	18.5
	Controls	59	16.3
Adjustment for inhalation			+

B.—Inhalation in relation to number of cigarettes per day*

Number of cigarettes per day	Number of cases		Percent of smokers who inhale	
1–9	Cancer	31	13	
	Controls	40	18	
10–19	Cancer	67	64	+
	Controls	58	45	
20–29	Cancer	63	65	
	Controls	44	50	
30+	Cancer	15	67	
	Controls	7	57	
Adjustment for number of cigarettes per day				+

*See footnote, table 10.

association between inhalation and these diseases. (*b*) Inhalation is individually associated with cancer, independently of these diseases.

The most interesting disease is chronic bronchitis, which is much more frequent among lung cancer patients than among controls. Analysis shows that lung-cancer is associated with: (*a*) chronic bron-

TABLE 13.—Cancer of the lung: method of smoking and inhalation (smokers)*

A.—Inhalation in relation to method of smoking*

Method of smoking	Number of cases		Percent of subjects who inhale†
Cigarette only	Cancer	1,039	59+++
	Controls	774	43
Cigarette and pipe or pipe only	Cancer	59	20
	Controls	138	13
Adjustment for method of smoking			+++

B.—Method of smoking in relation to inhalation*

Inhalation†	Number of cases		Percent of cigarette-only smokers
No	Cancer	471	90+++
	Controls	560	79
Yes	Cancer	627	98++
	Controls	352	95
Adjustment for inhalation			+++

*Smokers include cigarette-only, cigarette-and-pipe, and pipe. Cigarette smokers include cigarette-only and cigarette-and-pipe. Significance is noted +, ++, +++, for $P = 0.05$, 0.01, 0.001, and below.

†Cigarette or pipe smoke is taken into account for cigarette-only or pipe-only smokers. *Both* are taken into account for mixed smokers.

TABLE 14.—Cancer of the larynx: method of smoking and inhalation (smokers)*

A.—Inhalation in relation to method of smoking*

Method of smoking	Number of cases		Percent of subjects who inhale†
Cigarette only	Cancer	220	61+++
	Controls	168	43
Cigarette and pipe or pipe only	Cancer	17	29+
	Controls	33	6
Adjustment for method of smoking			+++

B.—Method of smoking in relation to inhalation*

Inhalation†	Number of cases		Percent of cigarette-only smokers
No	Cancer	97	88+
	Controls	127	76
Yes	Cancer	140	96
	Controls	74	97
Adjustment for inhalation			+

*Smokers include cigarette-only, cigarette-and-pipe, and pipe smokers. Cigarette smokers include cigarette-only and cigarette-and-pipe. Significance is noted +, ++, +++, for $P = 0.05$, 0.01, 0.001, and below.

†Cigarette or pipe smoke is taken into account for cigarette-only or pipe-only smokers. *Both* are taken into account for mixed smokers.

chitis, regardless of whether the subject inhales or not; (*b*) inhalation, regardless of whether the subject has had bronchitis; however, when the subject has had this, the effect of inhalation is hardly noticeable.

DISCUSSION

The foregoing study enables us to list cancers associated with smoking, which include at least the following sites: lip, tongue, oral cavity, oro-

TABLE 15.—Cancer of the bladder: method of smoking and inhalation (smokers)*

A.—Inhalation in relation to method of smoking*

Method of smoking	Number of cases		Percent of subjects who inhale†
Cigarette only	Cancer	176	56++
	Controls	149	40
Cigarette and pipe or pipe only	Cancer	12	17
	Controls	18	0
Adjustment for method of smoking			++

B.—Method of smoking in relation to inhalation*

Inhalation†	Number of cases		Percent of cigarette-only smokers
No	Cancer	88	89
	Controls	108	83
Yes	Cancer	100	98
	Controls	59	100
Adjustment for inhalation			

*Smokers include cigarette-only, cigarette-and-pipe, and pipe smokers. Cigarette smokers include cigarette-only and cigarette-and-pipe. Significance is noted +, ++, +++, for $P = 0.05$, 0.01, 0.001, and below.

†Cigarette or pipe smoke is taken into account for cigarette-only or pipe-only smokers. *Both* are taken into account for mixed smokers.

pharynx, hypopharynx, larynx, lung, esophagus, and bladder. This list coincides with those obtained by the prospective surveys of Hammond and Horn (*8*) and Dorn (*9*). The interest of this confirmation lies in the fact that our results are obtained through a different approach: a retrospective survey on all cancers with matched controls, each pair being questioned by the same interviewer.

Special mention should be made of cancer of the prostate, which, in these prospective surveys, was a cause of higher mortality among smokers of cigarettes. We have independently observed a relation to the use of cigarettes, but with such a slight significance that it may not be worth considering.

Among cancers associated with smoking, two groups appear: (*a*) In cancer of the lip, oral cavity (excluding the tongue), oropharynx, hypopharynx, and esophagus, inhalation and a preference for cigarettes seemingly are not noteworthy. The peculiar problem of cancer of the tongue remains to be explained. (*b*) In cancer of the lung and larynx, and most likely bladder, inhalation and a preference for cigarettes do have a significance.

In studying these three sites we have considered three criteria: amount smoked, method of smoking, and inhalation. We studied the significance of each of these when the other two simultaneously were controlled. It appeared that: (*a*) *The amount smoked* is a factor in cancer of the lung, larynx, and bladder (tables 10A, 11A, 12A); (*b*) *inhalation* also is a factor in relation to these three sites (tables 10B, 11B, 12B); (*c*) *method of smoking* (cigarette smoking) is a factor in cancer of the lung (table 7B); this significance is hardly, or not at all, noticeable in cancer of the larynx and bladder (tables 14B and 15B).

The importance of these last two criteria is worth examining more closely:

(1) The *effect of inhalation* has been contested. Notably in the study of Doll and Hill (*10*), the proportion of subjects inhaling was not higher among lung-cancer patients than among controls. Fisher (*11*) saw in this a serious objection to a causal interpretation of the effect of tobacco. To the contrary, we have already indicated the importance of inhalation in our earliest study (*1*).

It now appears to us that this is entirely confirmed: (*a*) We believe that the habit of inhaling has been correctly evaluated among our patients. The relationships between this habit and the amount smoked, the method of smoking, or the subject's age are very consistent and in agreement with previous observations (*4*, *5*). The relationship between inhalation and the advent of diseases like arteriosclerosis, some lung diseases, and various symptomatic disorders has also been another confirmation.

(*b*) The influence of inhalation in regard to these three sites is confirmed by a very strong significance, at least for the lung and the larynx. The significance persists even when sampling factors and other criteria of consumption are considered.

(*c*) The fact that such a significance appears in cancer of the lung, larynx, and even bladder, and not in connection with the other sites, is relatively consistent from the physiopathological viewpoint.

This effect of inhalation, in the final analysis, seems to be well established. That the effect is particularly clear in our own study could result from the fact that the population we studied comprised a fairly large proportion of average and light smokers, in whom the effects of inhalation are more pronounced, and from the choice of a healthy control group.

(2) *The significance of cigarettes* seems less important to us than that of inhalation: When inhalation is controlled, the particular role of cigarettes fades; it is hardly apparent in cancer of the larynx and the bladder; it is clear only in lung cancer. This may be caused by the very great number of subjects. It is therefore certain that the results of cigarette smoking are mostly from the habit of inhaling.

The habit of inhaling, however, does not entirely explain the significance of cigarette smoking, since the latter is still independently apparent among subjects who do not inhale.

Finally, this residual role calls for an explanation; certain peculiarities of the cigarette have been alleged to cause this, chiefly the paper. But the effect of such factors, even if they exist, cannot provide a sufficient explanation. The high risk of smokers of the cigarette-only category, as opposed to those of the "mixed" category, remains just as high when both categories smoke the same number of *cigarettes* (and even when subjects do not inhale). An explanation must therefore be sought in the smoker himself. A reasonable interpretation of the facts might be that smokers of the cigarette-only, pipe-only, and "mixed" categories, who already present noteworthy differences in regard to inhalation, also differ in their general manner of smoking (frequency, duration, and depth of the puffs, etc.).

APPENDIXES

I. Study of Ill Controls

As previously mentioned, 1,807 pairs of male controls were studied. Each pair was composed of one *ill* and one *healthy subject* (work or traffic accident victim), matched by age (5-year-age group), interviewer, period of interview, and group of hospitals. The ill subjects were hospitalized in general medical wards for various diseases. The only disease excluding a patient from the survey was active tuberculosis. Some of these diseases might be related to the use of tobacco, and especially to inhalation, which could explain the difference between ill and healthy subjects, shown in table 1.

To verify this, we classified ill subjects according to cause of hospitalization, following the ISC classification of diseases, and used the first two figures. We considered only illnesses represented by a minimum of 25 patients. The various groups thus constituted were compared to the corresponding healthy men. The results are given in table 16.

While adopting ISC's classification of diseases, which designates important groups, by the first 2 digits of a 3-digit number, the third digit dividing each group into categories, we have used the first 2 digits together only with (*a*) the group designation, whenever our controls came under more than one category in a group, or (*b*) the category title whenever our controls came under one category only in a group, *i.e.*, in group 32, "disorders of character, behavior, and intelligence," all our controls belonged to category 322, "alcoholism," and thus are listed, 32: alcoholism.

Of the 21 causes of hospitalization listed in this table, 6 show a higher rate of inhalation than is found among healthy subjects, in a significant, or nearly significant proportion: chronic alcoholism, coronary heart disease, arterial disorders (chiefly, generalized arteriosclerosis), pneumonia, bronchitis, and diverse symptomatic disorders related to various systems (in fact, chiefly, the cardiovascular, the respiratory, and the digestive systems).

These 6 types of diseases excepted, the other ill controls showed practically no difference from their healthy counterparts; this can be seen in table 17 in which ill subjects are classified in 2 groups: (*a*) those who were hospitalized for 1 of the 6 diseases given. These differ considerably from the corresponding healthy controls (proportion of subjects inhaling, 54% against 37%); (*b*) those who were hospitalized for some other reason. These no longer differ significantly from their controls (47% as opposed to 43%).

In support of this separation into 2 groups we bring two arguments: 1) The diseases of (*a*) are precisely those in which the effects of smoking have been noted, or appear plausible (*12*); it should be recalled in particular that coronary heart disease, arteriosclerosis, pneumonia, and bronchitis contributed to the excess mortality of cigarette smokers in the prospective surveys (*8*, *9*). 2) We have also been able to confirm independently the relationship between inhalation and the diseases of (*a*). Through our questionnaire we had obtained information on the past medical history of the patients, which helped us to determine whether

TABLE 16.—Comparison between ill and healthy controls in regard to inhalation of cigarette smoke

Cause of hospitalization (ill controls)		Number of subjects in each of the 2 control groups	Percent subjects inhaling cigarette smoke		
ISC number	Designation (*see* text)		Ill controls	Corresponding healthy controls	Significance $P =$
24	Allergic diseases	35	61	37	
26	Diabetes	32	25	14	
32	Alcoholism	69	66	43	0. 05
33	Vascular lesions affecting central nervous system	26	20	16	
35	Various diseases of the central nervous system	46	45	39	
42	Coronary heart disease	112	48	35	0. 07
43	Various heart diseases	188	41	39	
44	Hypertensive disease	39	43	34	
45	Diseases of arteries	52	66	33	0. 01
46	Diseases of veins	53	46	48	
48	Influenza	25	35	46	
49	Pneumonia	68	50	33	0. 08
50	Bronchitis	72	60	36	0. 02
51	Various diseases of the upper respiratory tract	29	38	54	
52	Various lung diseases	62	47	50	
54	Diseases of stomach and duodenum	69	48	40	
58	Diseases of liver, gallbladder, and pancreas	223	51	46	
59	Nephritis and nephrosis	30	29	57	
72	Arthritis and chronic rheumatism	55	37	39	
78	Various symptomatic disorders	166	52	38	0. 05
79	Various ill-defined diseases	29	56	33	

the tendency to inhale was stronger among patients who, in the past, had suffered from 1 of the 6 diseases of this group. After eliminating the subjects hospitalized because of these diseases, we compared the remaining ill and corresponding healthy controls (or, more exactly, pairs where the 2 controls are cigarette smokers).

The results are shown in table 18. The only valid comparisons concern the matched ill and healthy controls in each of the 4 cases. From one case to another, comparisons are not possible because the age of the

TABLE 17.—Comparison between ill and healthy controls in regard to inhalation of cigarette smoke

Cause of hospitalization of ill controls	Number of subjects in each of the 2 control groups	Percent of subjects inhaling cigarette smoke		
		Ill controls	Corresponding healthy controls	Significance* $P =$
One of the 6 diseases enumerated (*see* text) = group (*a*)	539	54	37	+++
Another disease = group (*b*)	1, 268	47	43	

*Significance is noted +++ for $P = 0.001$ and below.

subjects and the interviewer differs as well as the year of interview (the past medical history has been recorded with more of less detail according to the year). Ill and healthy controls are comparable when they have similar past medical histories. On the contrary, when past medical histories are not the same, the tendency to inhale is greater among controls with a medical history including a group (*a*) disease, regardless of whether these controls are the ill ones (case 2) or the healthy ones (case 3).

Finally this result is confirmed by our study of cancer patients. As can be seen in the section "Inhalation and previous diseases" under "Detailed Analysis" the tendency to inhale is greater among lung cancer patients who have had previous diseases of group (*a*).

The foregoing is but a schematic analysis. We do not claim that the 6 diseases listed constitute an inventory of diseases related to inhalation. For accuracy we have adopted a classification of diseases, using the first two figures of the international classification, which is systematic and objective, occasionally requiring subdividing or regrouping for our purpose.

TABLE 18.—Comparison between ill and healthy controls with regard to inhalation of cigarette smoke*

	Case 1		Case 2		Case 3		Case 4	
	Ill controls	Healthy controls	Ill controls	Healthy controls	Ill controls	Healthy controls	Ill controls	Healthy controls
Past medical history	0	0	P	0	0	P	P	P
Number of cases	216	216	179	179	135	135	240	240
Percent subjects inhaling cigarette smoke	46	47	51	40	33	45	50	47
Significance: $P=$			0.05		0.05			

*Subjects are marked P when they have suffered in the past from a disease of group (a), and 0 when they have not.

Yet certain disorders related to inhalation may have escaped our attention because of an insufficient number of subjects: This could be true, for instance, with group 79, close to 78, in which the tendency to inhale is also great. It is therefore likely that group (*b*) of ill subjects still includes certain diseases related to inhalation; by weeding these out, one would lower the slight difference between this group and its corresponding healthy controls (47% of subjects inhaling, as opposed to 43%).

Patients with diseases of group (*a*) are distinguished by the tendency to inhale much more than by any other criterion of consumption. Arterial disorders deserve special mention here: The corresponding subjects, compared to their healthy controls, do not differ by the proportion of smokers (83 against 75%) or by the amount smoked (12.8 ecd against 13.4). But there is a very great difference in regard to inhalation (66 against 33%). This is hardly surprising when one considers that the quantity of nicotine absorbed is nearly 10 times greater when the smoke is inhaled (*13*).

II. Inhalation and Previous Diseases in Lung Cancer

Table 19 indicates 4 possible comparisons between smokers among cancer patients and controls, according to whether smokers of either group have in the past suffered from a disease of type (*a*). The only valid comparisons deal with matched cancer patients and controls in each of the 4 cases. From one case to another, comparisons are not possible because the age of the subjects, interviewer, and year of interview differ (the past medical history has been recorded with detail according to the year). It appears that: 1) The tendency to inhale is in any event higher among cancer patients. 2) The difference becomes more important, or less important, according to whether the cancer patient or the control has had one of these diseases. This confirms the relationship of these diseases to inhalation. 3) These diseases occur more frequently among patients with lung cancer (53 times in 100) than among controls (41 times in 100). The main difference may be attributed to chronic bronchitis, which occurs with particular frequency among lung-cancer patients. Hence it is of interest to make clear whether bronchitis and inhalation are independently significant.

TABLE 19.—Comparison between lung-cancer cases and controls, in relation to past medical history*

	Case 1		Case 2		Case 3		Case 4	
	Cancers	Controls	Cancers	Controls	Cancers	Controls	Cancers	Controls
Past medical history	0	0	P	0	0	P	P	P
Number of cases	265	265	224	224	131	131	215	215
Percent subjects inhaling	62	39	63	36	49	45	57	44
Significance: $P=$	Highly significant		Highly significant				0.01	

*Subjects are marked P when they have suffered in the past from a disease of group (*a*), and 0 when they have not.

Table 20, A and B, show that: (*a*) chronic bronchitis is a factor regardless of whether or not the subject inhales; and (*b*) inhalation is a factor, at least when the subject has not had chronic bronchitis; when he has had, the role of inhalation is hardly noticeable. The foregoing analysis concerns smokers. One may ask whether chronic bronchitis has an effect on nonsmokers. We have not noticed such, but we were dealing with a small number of subjects.

TABLE 20.—Lung cancer: inhalation and chronic bronchitis among cigarette smokers

A.—Part played by chronic bronchitis in relation to inhalation*

Inhalation	Number of cases		Percent of subjects having had bronchitis: Raw	Adjusted for amount smoked
No	Cancer	465	33	+++
	Controls	516	17+++	
Yes	Cancer	637	33	++
	Controls	355	22+++	

B.—Part played by inhalation, with chronic bronchitis controlled*

Chronic bronchitis	Number of cases		Percent of subjects who inhale: Raw	Adjusted for amount smoked
No	Cancer	742	58	+++
	Controls	704	39+++	
Yes	Cancer	360	58	
	Controls	167	47+	

*Significance is noted +, ++, +++, for $P = 0.05$, 0.01, 0.001, and below.

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Myeloma Globulins of Plasma-Cell Neoplasms in Inbred Mice. I. Immunoelectrophoresis of Serum, With Rabbit Antibodies Prepared Against Microsome Fractions of the Neoplasms[1]

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SUMMARY

With the technique of agar-gel immunoelectrophoresis, a study was made of serum from normal BALB/c mice and from BALB/c mice bearing different plasma-cell neoplasms. The precipitating antibody used was developed in rabbits after immunization with washed microsomes obtained from transplants of the plasma-cell neoplasms. Three different neoplasms were employed separately: MPC-1, MPC-2, and Adj-PC-5. Each of these antibodies formed different patterns of precipitation with electrophoresed normal serum. Two arcs were developed: One, designated System I (SI), appeared to be identical with γ_2-globulin, and the second, designated System II (SII), was a β-globulin, the probable murine counterpart for the β_{1A}-globulin of man. The myeloma globulins appeared as anomalies of the SI and SII arcs in the form of crescent-shaped precipitates. The myeloma globulins of 13 different plasma-cell neoplasms of strain BALB/c were studied; 2 belonged to SI, 9 to SII, 1 was antigenically related to both, and 1 produced an SI and SII myeloma globulin. A remarkable degree of physical heterogeneity of the myeloma globulins was observed among the various cases within the systems. Further, many of the anomalous arcs which characterized the myeloma globulins of individual neoplasms were asymmetrical and were interpreted to indicate that the myeloma globulins made by individual neoplasms were also heterogeneous. Two stable protein-producing transplant lines were established from a single primary host, Adj-PC-6.—J. Nat. Cancer Inst. 26: 1109-1137, 1961.

THE TRANSPLANTABLE plasma-cell neoplasms of inbred mice present a direct experimental approach to problems in protein synthesis and somatic-cell genetics (differentiation). These highly functional neoplastic cells have determined patterns of protein synthesis. When considered as a group they can form many varieties of related proteins, but each individual neoplasm is restricted to making characteristic types (*1*). The pattern of protein synthesis, once determined, is a stable, heritable characteristic of the transplant line established from the primary neoplasm.

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A biologic system consisting of multiple, transplantable plasma-cell neoplasms originating in an inbred strain of mice has specific attributes: 1) Genetic variation in the primary host as a determinant in the speciation of protein secretion by neoplastic plasma cells can be virtually eliminated. 2) A series of myeloma globulins derived from a common genetic background is available for comparative physicochemical and immunochemical analysis as an approach to the problem of antibody formation. 3) Transplants of the plasma-cell neoplasms provide a rich source of protein-secreting tissue.

In the experiments reported here advantage was taken of the third attribute. Microsomes were isolated from three of the plasma-cell neoplasms and were used to immunize rabbits. The antibody obtained was used in an immunoelectrophoretic study of normal and pathologic sera from strain BALB/c mice. When normal BALB/c serum, as antigen, was electrophoresed in agar gel and developed with the rabbit antimicrosome antibodies, two major precipitin arcs were found. For reasons presented later, these arcs, which are probably the murine counterparts of the immunoglobulins of man, γ_2- and β_{2A}-globulin, were designated System I (SI) and System II (SII).

When sera of mice bearing plasma-cell neoplasms were examined by immunoelectrophoresis with the antimicrosome antibodies, anomalous SI or SII arcs were found in all cases. Thirteen transplant lines, originating from 12 primary hosts, were studied. The extra transplant line was established from the original host of the plasma-cell neoplasm Adj-PC-6, which gave rise to two different protein-producing, stable transplant lines, A and C.

MATERIALS AND METHODS

Plasma-cell neoplasms.—The origin of the transplantable plasma-cell neoplasms MPC-1, -2, -3, and -4 and Adj-PC-2, -5, -6, and -7 have been described (*1–4*). Additional information regarding Adj-PC-6 is presented in this paper.

The original host of Adj-PC-6 was given an intraperitoneal injection of 0.3 ml of incomplete Freund's adjuvants and heat-killed staphylococci. Nine months later this mouse developed multiple nodules of plasma-cell tumor in various parts of the peritoneum. There was one large tumor mass of 1 to 1.5 cm diameter in the suprapancreatic region, a mass of 1 cm diameter in an ovary, and multiple small nodules of 1 to 2 mm diameter scattered throughout the mesentery.

Four lines were developed and transplanted in parallel from this original mouse: Lines A, B, and D were established from mice implanted with the mesenteric tissue and line C from the pancreatic mass. None grew from the ovarian mass. Lines A, B, and D were similar. Line C differed strikingly from lines A, B, and D. By examination of routine hematoxylin-eosin-stained tissue sections, all four lines were characteristic

plasma-cell neoplasms. Lines A, B, and D will henceforth be referred to as line A.

Several new plasma-cell neoplasms originating in BALB/c mice which had received intraperitoneal injections of mixtures of incomplete Freund's adjuvants and heat-killed staphylococci were also included. These neoplasms were designated Adj-PC-8, -12, and -15, and LPC-1. The methods of routine transplantation have been described (*1*). With the exception of the X5563 sera (strain C3H) which were used in one of the studies, all other plasma-cell neoplasms, sera, and microsomes were obtained from BALB/c mice.

Preparation of washed microsome fractions.—Microsomal fractions were prepared from MPC-1 and MPC-2 plasma-cell neoplasms harvested 14 days after transplantation. In each instance, the pooled tumors from several mice were rinsed in ice-cold 0.25 M sucrose and homogenized for 1 minute in 2 volumes of this medium. Cell breakage was essentially complete. Nuclei and mitochondria were sedimented from the homogenate by a single centrifugation of 10 minutes at 11,000 $\times$ *g* (rotor #296, International PR-2 centrifuge), resuspended in 0.25 M sucrose, and resedimented by centrifugation for 10 minutes at 20,000 $\times$ *g* (*5*). The supernatant fluids from these 2 runs were combined and filtered through coarse bolting-silk (16 mesh/cm) to remove floating shreds of fatty connective tissue. The filtrate was centrifuged for 90 minutes at 40,000 rpm (105,000 $\times$ *g*) in the #40 rotor of the Spinco Model L ultracentrifuge and the supernatant fluid removed. The pellets, which represented the crude microsome fractions, were resuspended by homogenization in 0.25 M sucrose containing 5 mM sodium phosphate, *p*H 7.5, and 0.5 mM magnesium chloride. The addition of the phosphate-magnesium buffer was designed to preserve the integrity of the ribonucleoprotein elements of the microsomes during the washing (*5*). The microsome suspension was centrifuged for 90 minutes at 105,000 $\times$ *g* and the supernatant fluid discarded. The pale-amber microsomal pellets were resuspended in 0.145 M sodium chloride to constitute the washed microsome fraction (Pw) which was later administered as antigen to rabbits. The Pw fraction from each type of neoplasm was divided into aliquots which were shell-frozen at −70° C and stored at −10° C until used.

A washed microsome fraction was also isolated from the pooled livers of several mice bearing the MPC-1 neoplasm. The method of preparation was identical with that just described, except that the initial sedimentation of nuclei and mitochondria was carried out at 5000 $\times$ *g* (*6*) and filtration of the mitochondrial supernatant fluid was omitted.

The microsome fractions were analyzed for total nitrogen (*7*), ribonucleic and deoxyribonucleic acid phosphorous [RNA P and DNA P, respectively, (*8*)], and lipide phosphorous [lipide P (*5*)]. The results are presented in table 1.

Immunization.—Albino New Zealand rabbits were immunized with water-in-oil emulsion, a mixture of 1 part complete Freund's Adjuvant

TABLE 1.—Properties of washed microsome fractions (Pw) from plasma-cell neoplasms and mouse liver

	Liver (BALB/c)	Plasma-cell neoplasm		
		MPC-1	MPC-2	Adj-PC-5
Wet weight of tissue (g)	3.4	2.3	3.6	10.0
Volume of Pw (ml)	6.0	3.5	5.0	15.0
Composition of Pw (μg/ml)				
Total nitrogen	1515	1895	2135	2765
RNA P	105	254	244	339
DNA P	3	11	13	14
Lipide P	128	82	118	106

(Difco Laboratories, Inc., Detroit, Mich.) and 1 part antigen (serum or washed microsome fraction) as shown in table 2.

Four ml of the serum-adjuvant mixtures and 2 ml of the microsome-adjuvant mixtures were injected intramuscularly into 4 sites in the upper and lower extremities; this was repeated 2 weeks later. Three weeks thereafter, 0.2 ml of antigen without adjuvant was given subcutaneously.

TABLE 2.—Origin and designation of rabbit antisera

Antigens injected into rabbits	Paper electrophoretic mobility of myeloma globulin (reference)	Designation of antiserum
Pool of serum, normal BALB/c mice	—	R3
Pool of serum, mice bearing MPC-3	β (*1*)	R1
Washed microsomes from:		
MPC-1	α-3 (*1*)	MPC-1 Pw
MPC-2	β-serum, γ Bence Jones (*1, 4*)	MPC-2 Pw
Adj-PC-5	γ (*3*)	Adj-PC-5 Pw

Immunoelectrophoresis.—The techniques of Grabar and Williams (*9*) and Courcon and Uriel (*10*) were used with modifications. We dissolved 1 percent suspension of agar [4] in 0.05 M Veronal buffer, *p*H 8.2, by heating it to 90 to 100° C with the aid of a combination hotplate and magnetic stirring device. When the agar solution was clear it was placed in a 54° C water bath. Clean glass plates were painted with 2 percent agar, diluted in distilled water, and placed on glass rods over a hotplate until the agar film was baked on. When the plates were cooled they were transferred to specially made plastic trays [5] (fig. 1), and the clear 1 percent agar solution was poured over the glass plates to the desired level. The sides of the tray contained two removable blocks which were removed when the agar had cooled and formed a firm gel; a bridge-template with

[4] Two commercial agars were used: Special Agar Noble, Difco Laboratories, Inc., Detroit, Mich., and Ionagar No. 2, Consolidated Laboratories, Inc., Chicago Heights, Ill.

[5] The authors wish to thank Mr. Arthur C. Scruggs of the Division of Research Services, Instrument Engineering and Development Branch, National Institutes of Health, for assistance in the fabrication of the tray-template device.

feet, identical in size to the removable blocks, was then inserted and the center wells were cut (fig. 1).

The tray was placed between two tanks containing buffer, and the front and back gates were removed. Gauze wicks soaked with buffer were then placed on the straight edges of the agar block and sealed with hot agar. Veronal buffer, *p*H 8.2, 0.05 M, $\Gamma/2 = 0.037$, was used. Electrophoresis was carried out at room temperature for 5 hours for the large plates and 2 to 3 hours for the small plates. For the large plates (5 × 7 in.) a constant current of 45 ma was used, and for the small plates (3 × ¼ × 4 in.) 200 v, with a voltage between the two wicks of 25 to 30 v. After electrophoresis the wicks were removed, discarded, and the front and back gates and template replaced. The trenches were cut and filled with antiserum.

The plates were transferred to a moist chamber in a refrigerator at 5° C or at room temperature. Photographs were made 24 to 48 hours after electrophoresis.

Ouchterlony technique.[6]—The technique of Allison and Humphrey (*11*) was used. The 1 percent agar was melted in .067 M Na phosphate buffer, *p*H 7.0, which contained approximately 1 mM Versene-100[7] and 1 mM sodium azide. The plates were developed in petri dishes at room temperature. The diameter of the wells was 6 mm and the intercircumferential distance between wells 4 mm.

Abbreviations and terminology.—The name of a plasma-cell neoplasm preceding the word *serum*, *e.g.*, MPC-2 serum, is to be read "serum from a mouse bearing the plasma-cell neoplasm MPC-2." The rabbit anti-microsome sera have the letters Pw following the name of the neoplasm, *e.g.*, MPC-2 Pw. The precipitin reactions in the immunoelectrophoretic and Ouchterlony plates usually involve mixtures of both antigens and antibodies. To simplify reference to these reactions, the reactants, antigens, and antibodies, respectively, are given in brackets, *e.g.*, [MPC-2 serum—MPC-2 Pw] which means a set of precipitin arcs that resulted from MPC-2 serum, confronted with rabbit anti-MPC-2 washed microsomes.

The terms System I (SI) and System II (SII) are used to describe the 2 normal serum-protein precipitin arcs developed by the Pw antisera. SI and SII are used in place of γ_2 and β_{2A} for several reasons: 1) The normal serum proteins γ_2 and β_{2A} are not a single molecular type but a collection of electrophoretically heterogeneous molecules with a common set of antigenic determinants. 2) The use of these terms avoids emphasizing the importance of electrophoretic behavior as a criterion for the classification of myeloma proteins, at the expense of other important criteria such as antigenic properties.

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[6] The authors wish to acknowledge gratefully the technical assistance of Mrs. Charlotte R. Boyce in the preparation of the Ouchterlony plates.

[7] Tetrasodium salt of ethylenediaminetetraacetic acid was purchased from Versenes, Inc. Framingham, Mass.

RESULTS

Precipitin Patterns of Normal Sera and Sera From Mice With Carcinomas and Sarcomas

MPC-2 Pw antiserum developed both SI and SII arcs with normal sera (figs. 2 and 3). SI was a long, asymmetrical precipitin arc which began at the cathodal end of the plate and extended beyond the well into the α-mobility region. SI, as developed by MPC-2 Pw antiserum, appeared as two closely positioned arcs which were most widely separated at their cathodal ends. In some, anodal splitting was seen, but this was most often obscured by the SII precipitate, with which SI fused. The anodal end of the SI arc never crossed the SII arc. This finding would be expected if both SI and SII proteins shared antigenic determinants.

SII was usually found to be a thin, comparatively small, symmetrical precipitin arc with a midpoint just anodal to the well (figs. 2 and 3). Most often the arc was single, but occasionally appeared to be a double arc. The SII arcs in [normal serum–MPC-2 Pw] reactions (fig. 2) showed considerable variation, particularly in the sharpness of precipitation. In normal sera the SII arc always developed closer to the antigen source than SI. Ten SII arcs in normal serum and serum from mice bearing other types of neoplasms are shown in figure 2.

MPC-2 Pw antiserum occasionally formed 2 faint arcs in the α- and albumin regions. Neither of these reactions appears in figures 2 or 3.

Adj-PC-5 Pw antiserum developed a pattern with normal serum that differed sharply (figs. 4 and 5). No SII arc was demonstrable. The SI pattern, instead of being a pair of nearly parallel arcs, was a single dense arc which separated into three lines anodally. There was a long, outer line that extended into the α-region, a short, inner line that ended near the well, and an intermediate line. The 3 lines were most prominent in the sera of hyperimmunized mice, and for illustrative purposes such a serum was used in experiments which are shown in figures 4 and 5. Other normal sera were qualitatively similar to the pattern just described.

MPC-1 Pw antiserum formed a moderately heavy precipitate with SII and only weakly developed SI arcs (figs. 6 and 7). The SII arc was similar to the SII arc developed by MPC-2 Pw antiserum. In plates where SI arcs did develop, it was the impression that a double arc system was demonstrable.

Comparison of Antibodies Developed Against Washed Microsomes and Whole Serum

The Pw antisera formed precipitates with only a few of the proteins of normal serum, principally SI and SII. This selectivity was best demonstrated when rabbit antibodies against serum were diffused from one trench and the Pw antiserum from the opposing trench (figs. 3 and 4).

The similarity of the SI precipitin arcs with γ_2 of [normal serum–R1] and [normal serum–R3] reactions was clearly demonstrable. The arc

of γ_2 is a characteristic arc for all mammalian species thus far studied (including mice) with the technique of immunoelectrophoresis (*12*, *13*). It is well known that the type of γ_2-arc formed in man is dependent on the antiserum used, for double arcs have been described by Edelman *et al.* (*14*) to develop with some antisera. The SI arcs of [normal serum–R1] and [normal serum–R3] were different. R1 antiserum developed a terminally split SI while R3 did not.

A clear example of an SII arc was not developed by either R1 or R3 antiserum. Such an arc may have developed but, if so, it was obscured by other heavier arcs. There was an ill-defined precipitate that developed in the [normal serum–R3] reaction roughly comparable in location to SII.

It may be concluded that without a selective antiserum such as MPC-2 Pw or MPC-1 Pw, it would be difficult to demonstrate the presence of SII in normal serum. Though the technique of immunoelectrophoresis cannot be used as a reliable quantitative method, it is justifiable to conclude that SII proteins are present in small amounts in normal serum.

General Characteristics of Myeloma Globulins

Immunologic.—Myeloma globulins appeared as regions along the SI (fig. 4) or SII (fig. 3) arcs where greatly increased quantities of antigen migrated during electrophoresis. The precipitates that formed around these regions were crescents that bowed toward the trenches (figs. 5 and 6). Some of the arcs were asymmetrical, particularly the anomalous SII. This resulted from a principal zone of protein concentration and one or more minor zones. An example is the MPC-2 SII arc in figure 3. The myeloma globulins represent a great increase in quantity of one or more molecular types within System I or System II. An antiserum against a myeloma globulin of one system strongly cross-reacts with all others within the system (figs. 5 and 6).

The myeloma globulins of 13 tumor transplant lines from strain BALB/c were grouped antigenically as follows:

SI only: Adj-PC-5, LPC-1 (X5563–C3H)

SII only: MPC-1-3, and -4, Adj-PC-2, -7, -8, -12, and -15

SI and SII: MPC-2, Adj-PC-6C, and tentatively Adj-PC-6A.

Electrophoretic mobility.—The case-to-case variation in electrophoretic mobility of the myeloma globulins is illustrated in figures 5 through 8.

A line perpendicular to the axis of electrophoresis and the midpoint of a crescent on an arc, at the trench, was considered the region of greatest electrophoretic concentration of the antigen and was an indication of the mean of the electrophoretic mobilities in agar of the principal component. Absolute mobilities were not determined. Patterns closely resembling each other were differentiated by side-by-side electrophoresis to show relative mobilities (figs. 7 and 8).

The technique was also valuable for a comparison of similarities. In figure 7, for example, MPC-1 serum from transfer generations 8 and 39 had identical patterns in the SII arc.

Extremes of electrophoretic migration were seen in System II (fig. 6).

The myeloma globulin of Adj-PC-7 was the most cathodal, while the myeloma globulins of MPC-1, MPC-4, and Adj-PC-12 were most anodal. Subtle differences were detected by side-by-side electrophoresis, *e.g.*, the differences in mobilities of MPC-1 and MPC-4, and the differences between MPC-2 and MPC-3 (fig. 8).

A similar gradient in electrophoretic mobilities was observed in the three examples of SI myeloma globulins LPC-1, Adj-PC-5, and X5563 (fig. 5).

Further Characteristics of Pw Antisera

Similarity of MPC-2 and R1 antisera in their abilities to develop the characteristic SII myeloma globulin arc in MPC-2 serum.—It was seen that SII myeloma globulins were well developed by MPC-2 Pw antiserum. R1 (anti-MPC-3 serum) developed a precipitin arc in MPC-2 serum which was similar to the one developed by MPC-2 Pw antiserum (fig. 3). The SII arc was interwoven with another prominent β-globulin (probably siderophilin) and anodally fused with albumin. R3 (antinormal serum) did not develop the MPC-2 myeloma globulin arc (fig. 3). This latter point is particularly striking in view of the large quantity of MPC-2 myeloma globulin in the serum. This may be explained as follows: The rabbit immunized with normal serum failed to develop anti-SII precipitins because of (*a*) competition of other strong antigens in serum, and (*b*) small amounts of SII in normal serum. The rabbit immunized with MPC-3 serum was given relatively large quantities of myeloma globulin and effectively formed anti-SII precipitins.

It was possible to absorb R1 with normal BALB/c serum and leave behind an antiserum which specifically developed only the MPC-2, MPC-3, and other SII arcs (fig. 9). The absorbed antiserum formed no line with BALB/c serum.

Failure of Adj-PC-5 Pw to precipitate SII myeloma globulins, and MPC-1 Pw antiserum to precipitate SI myeloma globulins.—Adj-PC-5 Pw failed to precipitate the SII myeloma globulins of Adj-PC-8 and -12, and MPC-1, -2, and -3 (figs. 5 and 10). In experiments to be reported elsewhere, an LPC-1 Pw antiserum reacted like Adj-PC-5 Pw, both in respect to the arcs formed with normal serum and by its failure to precipitate the SII myeloma globulin in MPC-2 serum.

MPC-1 Pw antiserum failed to precipitate the myeloma globulins of the SI series. This antiserum did not develop as much or as dense a precipitate with SII myeloma globulins as MPC-2 Pw antiserum (fig. 6). Very faint SI precipitin arcs were formed in [normal BALB/c serum–MPC-1 Pw] reactions. These facts may indicate that a quantitative rather than a qualitative difference exists between MPC-1 Pw and MPC-2 Pw.

Two Myeloma Globulins Derived From the Primary Host of Adj-PC-6

Separate nodules of plasma-cell neoplasm were established and transplanted in parallel from the primary host of Adj-PC-6. When sera from

the various transplant lines became available there were two sharply different protein-producing lines, A and C. It was concluded that the original host of Adj-PC-6 had developed two primary plasma-cell neoplasms that were distinguishable only by the proteins they made. Each transplant line maintained its own characteristics during serial transplantation.

The myeloma globulin of Adj-PC-6A precipitated around the well in the agar gel (0.05 M Veronal buffer, *p*H 8.2; figs. 6 and 11) and also in distilled water. The precipitation occurred on the cathodal side of the well when the serum migrated into the low ionic strength medium. It was not caused by antiserum for it was demonstrable during the progress of the electrophoresis. Some protein did diffuse away from this region and combine with antiserum.

No similar precipitation was ever observed with Adj-PC-6C serum. The precipitin arcs formed by the two myeloma globulins with MPC-2 Pw antiserum differed greatly in length and contour. The 6C arc clearly consisted of two components, a long crescent on the cathodal side of the well and a small crescent on the anodal side. The 6B arc developed around the region where the precipitate formed (fig. 11).

Myeloma Globulins With Both SI and SII Antigenic Determinants

MPC-2 Pw precipitated the myeloma globulin of both Adj-PC-6A and Adj-PC-6C. The myeloma globulin arc of Adj-PC-6C fused with the inner arc of SI (fig. 11). In contrast, the Adj-PC-7 myeloma globulin arc, which had a similar mobility, clearly separated from both lines of SI (fig. 6). It was concluded from this observation that the myeloma globulin of Adj-PC-6C contained SII, and at least one SI, antigenic determinants. A detailed description of the differences between these two lines will be published separately.

It has been previously described that the mice bearing the plasma-cell neoplasm MPC-2 have a serum myeloma globulin of β-mobility as well as a urinary Bence Jones protein of γ-mobility. Most MPC-2 sera show, in addition to the anomalous SII arc, a very definite but slight bowing of the SI arcs (figs. 3 and 6). This may be explained as small amounts of the Bence Jones protein (antigentically related to SI) in serum. The microsome fraction of the MPC–2 neoplasm, however, must also have contained these antigenic determinants. This may explain why MPC–2 Pw effectively formed both SI and SII arcs and may further explain the differences between MPC-1 Pw and MPC-2 Pw.

Ouchterlony Method

Normal BALB/c serum in varying dilutions was diffused in agar gel toward MPC-2 Pw antiserum (double-diffusion technique of Ouchterlony). Two lines formed with undiluted serum: a thick line close to the well and a line which formed half way between the two wells (fig. 12)

As the normal BALB/c serum was progressively diluted the outer line became ill defined at the 1:20 dilution and formed progressively closer to the antigen well (fig. 13). The inner line also became gradually thinner and sharper, but persisted even at dilutions of 1:100 (fig. 13). The inner line formed close to the center well (antibody) when the antigen was undiluted, but formed midway between the two wells at 1:40 dilution of normal serum. A suggestion of two lines was noted for the inner precipitation line at antigen dilution 1:50 (fig. 13).

The MPC-1 Pw antiserum also formed an outer and inner line with normal BALB/c serum (fig. 16). The inner line was faint and ill defined, while the outer line was quantitatively similar to the [normal BALB/c serum–MPC-2 Pw] outer line.

When myeloma sera were used as antigens, the inner or outer lines persisted through much greater dilution (figs. 14, 15, and 17). This permitted identification of the two precipitin lines. The inner line was the same as the SI and the outer the same as the SII as revealed by the immunoelectrophoretic patterns.

When specific reactions were examined, *e.g.*, [MPC-2 serum-MPC-2 Pw] reaction and [MPC-1 serum–MPC-1 Pw] reaction, the outer line, SII, was the only one that persisted through dilutions greater than 1:60 and at these dilutions the outer line divided into separate, sharp lines (figs. 15 and 17). The new lines appeared on the antigen side of the outer line for the [MPC-2 serum–MPC-2 Pw] and [MPC-1 serum–MPC-1 Pw] reactions and required 48 hours for development. The ends of the line clearly ran into both normal antigen wells for the first reaction, which indicated that normal serum diluted 1:5 was almost or completely deficient in this substance.

Another form of splitting was noted in the two reactions mentioned here, *i.e.*, the ends of the outer line bifurcated as they approached the normal antigen well.

DISCUSSION

Twelve plasma-cell neoplasms that gave rise to 13 stable transplant lines were examined by agar-gel immunoelectrophoresis, with the use of antimicrosome antibodies. All the serum samples of each transplant line submitted to immunoelectrophoresis gave similar patterns, though they were obtained from mice bearing different transplant generations. The neoplasms appeared in strain BALB/c mice after intraperitoneal implantation of a Millipore diffusion chamber or after injection of adjuvant-staphylococcal mixtures. It was shown again that the myeloma globulins of plasma-cell neoplasms which share a common genetic background may differ widely in their electrophoretic characteristics. This observation was reinforced by the fact that 2 different myeloma-globulin-producing transplant lines were isolated from one original host.

It was also demonstrated that myeloma globulins from a single strain of mouse differed immunologically as well as electrophoretically. Based

on the technique of immunoelectrophoresis, immunologic differences between myeloma globulins in the mouse (*15*) and man (*16–18*) have been described previously. In man, there are two basic immunoelectrophoretic types of myeloma globulins, the γ_2 and β_{2A}; these are analogous to the two systems described here, SI and SII, respectively. In normal serum the SI arc fused with SII and the anodal end of SI never crossed SII. There are common antigenic determinants in these two systems of proteins as well as antigenic determinants unique to each one. A myeloma globulin might possess antigenic determinants of SI or SII. On the other hand, it might have antigenic determinants shared by both systems. The 13 myeloma globulins were classified into 3 groups: SI (2 cases), SII (8 cases), and SI + SII (3 cases). Within the SI and SII groups, the myeloma globulins had a wide range of different electrophoretic mobilities.

A series of myeloma globulins from plasma-cell neoplasms developed in a single inbred strain of mice provides an important genetic control for the comparative immunologic analysis of myeloma globulins. Without this control it would be difficult to establish whether the presence or absence of an antigenic determinant on a myeloma globulin molecule was real or an artifact created by a nonspecific heterologous antiserum. It has been shown that there are serum protein allotypes in rabbits (*19*) and, further, that different γ-globulin isoantigens (*20*) in randombred populations of rabbits are under genetic control (*21*).

Aside from the immunologic and electrophoretic heterogeneity of myeloma globulins in the 13 cases, evidence for another type of heterogeneity was observed—the heterogeneity of myeloma globulins produced by a single neoplasm. Evidence for this was obtained from the dimensions and asymmetry of the abnormal arcs. These arcs covered a great distance on the plate and the contour contained dips and curves on the inner (antigen) surface. The arcs differed from the symmetrical arcs of proteins such as siderophilin and albumin.

Examples of arcs showing asymmetry are the SII arcs of MPC-2 and -3, and the Adj-PC-6C and -7. Further evidence for heterogeneity of the myeloma globulins of mice has been obtained by Fahey *et al.*, who found components with sedimentation coefficients of 9, 11, and 13S in the serum of mice bearing the plasma-cell neoplasm X5647 (*22*). Askonas has recently found the myeloma globulin of X5563, which appears as a single band on paper electrophoresis, has 5 discrete bands in starch-gel electrophoresis (*23*).

Interpretation of the individual heterogeneity is difficult. Such heterogeneity may reflect a disorder of protein synthesis within the neoplastic plasma cells or may be a manifestation of a normal physiologic process. For example, the following argument might be made on the basis of existing knowledge of Bence Jones proteins: These proteins have been considered to be fragments of certain immunoglobulins (*24*) and are well known to be heterogeneous in their antigenic (*24*) and physicochemical properties (*25*). The heterogeneity of the myeloma

globulins may be the result of a disorder in the organization of fragments which ordinarily compose the normal immunoglobulins.

However, this heterogeneity may actually be characteristic of the normal antibody molecules. Unfortunately we do not have the important link of evidence to show that myeloma globulins carry specific antigen-combining sites. Even if this cannot be shown, the electrophoretic studies of myeloma globulins may still be revealing characteristics of antibody molecules other than those related to the specific antigen-combining site, *e.g.*, characteristics of some carrier portion of the molecule. The physicochemical variants might then represent a group of different carrier molecules that could provide greater versatility for combination with antigens under different physical, chemical, and pathophysiologic conditions.

The development of two stable transplant lines from one host was the first such example found in our experience. There are difficulties in isolating several lines from a single host: 1) Not all plasma-cell neoplasms grow when transplanted; 2) not all transplantable plasma-cell neoplasms grow in all isologous recipients (*3*); 3) the plasma-cell neoplasms that develop after the injection of adjuvant-staphylococcal mixtures are associated with ascites, which indicates, as the histologic sections also suggest, seeding of one vigorously growing population over others. Thus when nodules are selected for transplanting, contamination with a rapidly growing line results in overgrowth by the more vigorous population. In future experiments it will be important to develop the optimal mode of transplantation for primary plasma-cell neoplasms. Such a method may reveal many different protein-producing "clones" or "primary" plasma-cell tumors which develop simultaneously in a single host.

Washed microsome fractions from plasma-cell neoplasms carried antigenic sites related to those of the proteins composing the SI and SII systems in the serum. Whether this association was the result of secondary adsorption of the myeloma proteins occurring upon homogenization of the tissue, or whether it was related to a protein synthetic activity originally carried out by the microsomal structures is not known. Kern *et al.* (*26*) have shown that specific antibody activity can be detected in microsome fractions isolated from the lymph nodes of immunized guinea pigs and have presented evidence that this association is not simply the result of adsorption of antibody from the soluble fraction of the homogenates. Similarly, a number of other specific protein-secretory products have been found in association with microsome fractions derived from their respective tissues of origin (*27–30*). Askonas has found the X5563 myeloma globulin in microsomes and has separated it from the microsomes. Of great interest was her demonstration that all five bands were observed on starch-gel electrophoresis after release of the protein from the microsomes (*24*). In experiments to be described later, we observed that rabbits formed precipitating antibodies against the SI and SII serum proteins after injections of ribonucleoprotein (RNP) fractions isolated from MPC-1 and MPC-2 microsomes by deoxycholate treatment. This

observation is particularly analogous to the finding of Siekevitz and Palade (*31*) that similarly prepared RNP fractions of pancreas carried several of the enzymatic activities which appear in the pancreatic secretion. However, a detailed interpretation of the present findings in terms of the site and mechanism of myeloma protein synthesis within the neoplastic plasma cells must await the results of further work.

The technique of using microsomes from a uniform cell population for development of precipitins against serum proteins may have value in finding the cellular origin of other serum proteins.

* * *

The authors are grateful to Dr. Elliott F. Osserman, Francis Delafield Hospital, College of Physicians and Surgeons, New York City, for demonstrating to them the technique of immunoelectrophoresis and all its pertinent details.

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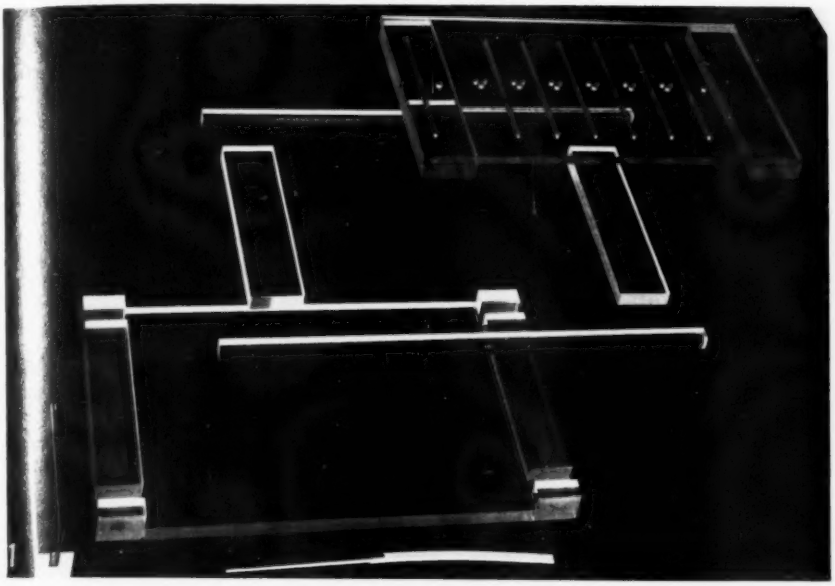
PLATES

PLATE 148

FIGURE 1.—Tray and accessories (exploded view). Plastic tray template for 3¼ × 4-inch glass slides. *Lower level:* base of tray, well cutter, and trench cutter. *Middle level:* removable front and back gates which fit in grooves; two end rectangular panels equal in size to feet of template. *Upper level:* template. Glass plate fits between gates and after agar is poured and hardened, the panels and front gate are removed. Template that bridges agar is then put in place.

FIGURE 2.—Immunoelectrophoresis of 10 different serum samples from BALB/c mice. Antibody throughout was rabbit anti-MPC-2 Pw serum. Antigens, in round wells, from *left* to *right:* 1 through 4 = normal frozen and thawed serum; 5 and 6 = serum from mice with MCA-induced skin carcinomas; 7 through 10 = serum from mice transplants of MS-1, AMT-2, MS-2, and AMT-1, respectively. Cathodal pole of plate faces top of page. (MS-1 and MS-2 are 2 transplantable fibrosarcomas of strain BALB/c, and AMT–1 and AMT–2 are 2 transplantable agent-containing mammary tumors of strain BALB/c.)



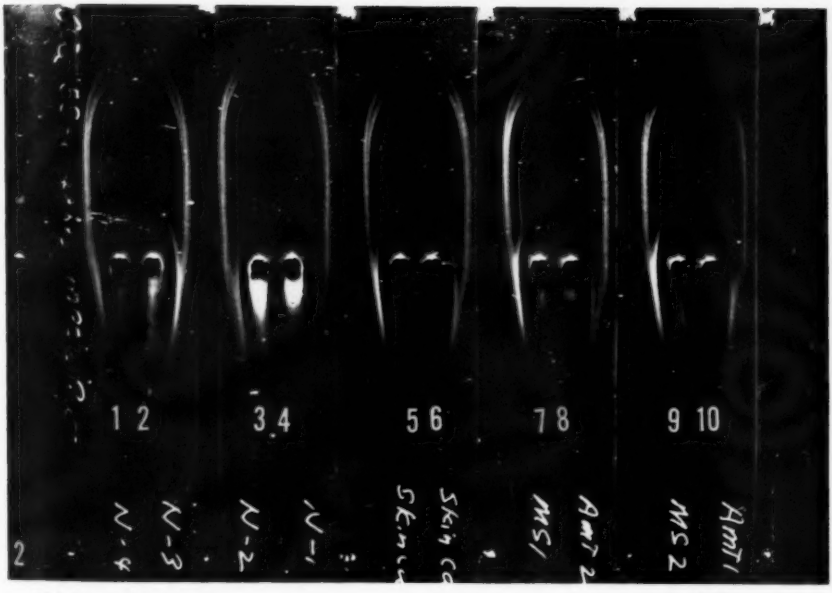


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PLATE 149

FIGURE 3.—Comparison of MPC-2 Pw antiserum [trench (t) 1, 3, and 5] with R1 (t 4) and R3 (t 2) antisera. Normal BALB/c serum [well (w) 1 and 3] and MPC-2 serum (w 2 and 4) were electrophoresed. Characteristic SI and SII arcs of normal serum precipitated by MPC-2 Pw are indicated by arrows over w 1. *Arrow* over w 2 points to precipitin arc formed by both R3 and R1 (w 4) with MPC-2 serum. Similar arc was not precipitated in normal serum by either antiserum, nor did arc appear in MPC-2 serum precipitated by MPC-2 Pw. This arc may represent substance which appears in serum in response to growing neoplasm but is not formed in neoplasm. *Arrow* over w 4 points to myeloma globulin arc of MPC-2 precipitated by R1. Anodally this arc fuses with albumin.

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PLATE 150

FIGURE 4.—Comparison of Adj-PC-5 Pw antiserum (t 1, 4, and 6) with MPC-2 Pw (t 2), R1 (t 5), and R3 (t 3). Normal serum (w 1, 3, and 5) and Adj-PC-5 serum (w 2 and 4) were electrophoresed. *Arrow* over w 1 indicates characteristic SI arc precipitated in normal serum by Adj-PC-5 Pw; *note* absence of SII.

FIGURE 5.—Differences in electrophoretic mobility of 3 SI myeloma globulins X5563 (w 3), Adj-PC-5 (w 4), and LPC-1 (w 5), which were precipitated by Adj-PC-5 Pw (t 2, 3, 4, and 5). For comparison, hyperimmune serum (w 2) and MPC-2 serum (w 1) were also electrophoresed, and MPC-2 Pw antiserum was placed in t 1 and t 6. *Note* Adj-PC-5 Pw did not precipitate the myeloma globulins in MPC-2 serum. MPC-2 Pw weakly precipitated LPC-1 myeloma globulin.

S1
1
1
2
3
4
5
FIG 4 84/2

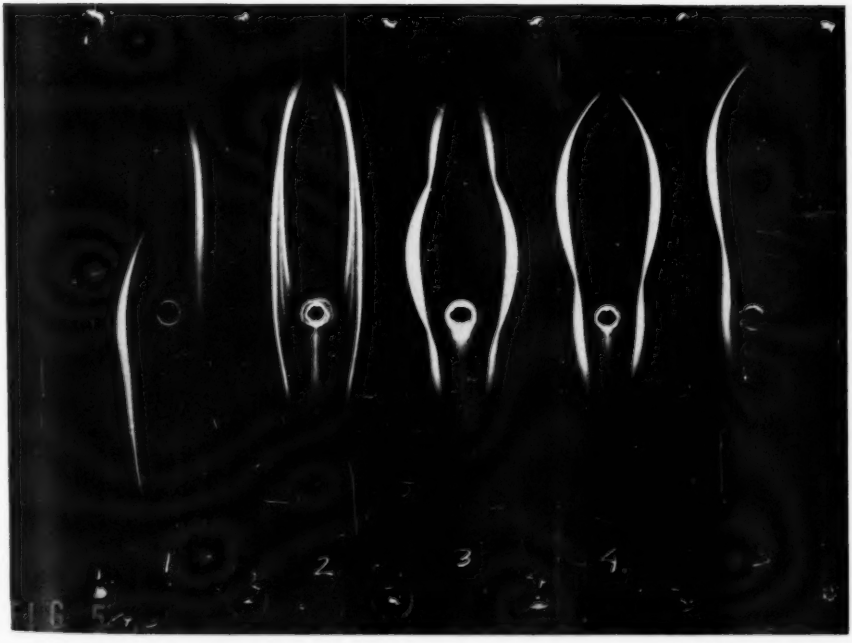

1
2
3
4
FIG 5

PLATE 151

FIGURE 6.—Variation in electrophoretic mobility and shape of myeloma globulin arc of 8 different plasma-cell neoplasms: Adj-PC-15 (1 w), Adj-PC-8 (2 w), Adj-PC-7 (w 3), Adj-PC-6A (w 4), Adj-PC-2 (w 5), MPC-3 (w 7), MPC-2 (w 8), and MPC-1 (w 9). Normal serum was placed in w 6. Antisera were MPC-2 Pw (t 1, 2, 4, 7, and 9) and MPC-1 Pw (t 3, 5, 6, 8, and 10). *Note* SII arc in Adj-PC-7 serum clearly separated from both SI arcs. Precipitate around Adj-PC-6A well appeared during electrophoresis and was not caused by antiserum. *Note* double arc developed by MPC-1 Pw with Adj-PC-6A. *Arrow* in t 9 points toward SI crescent that appeared in [MPC-2 serum–MPC-2 Pw] reaction.

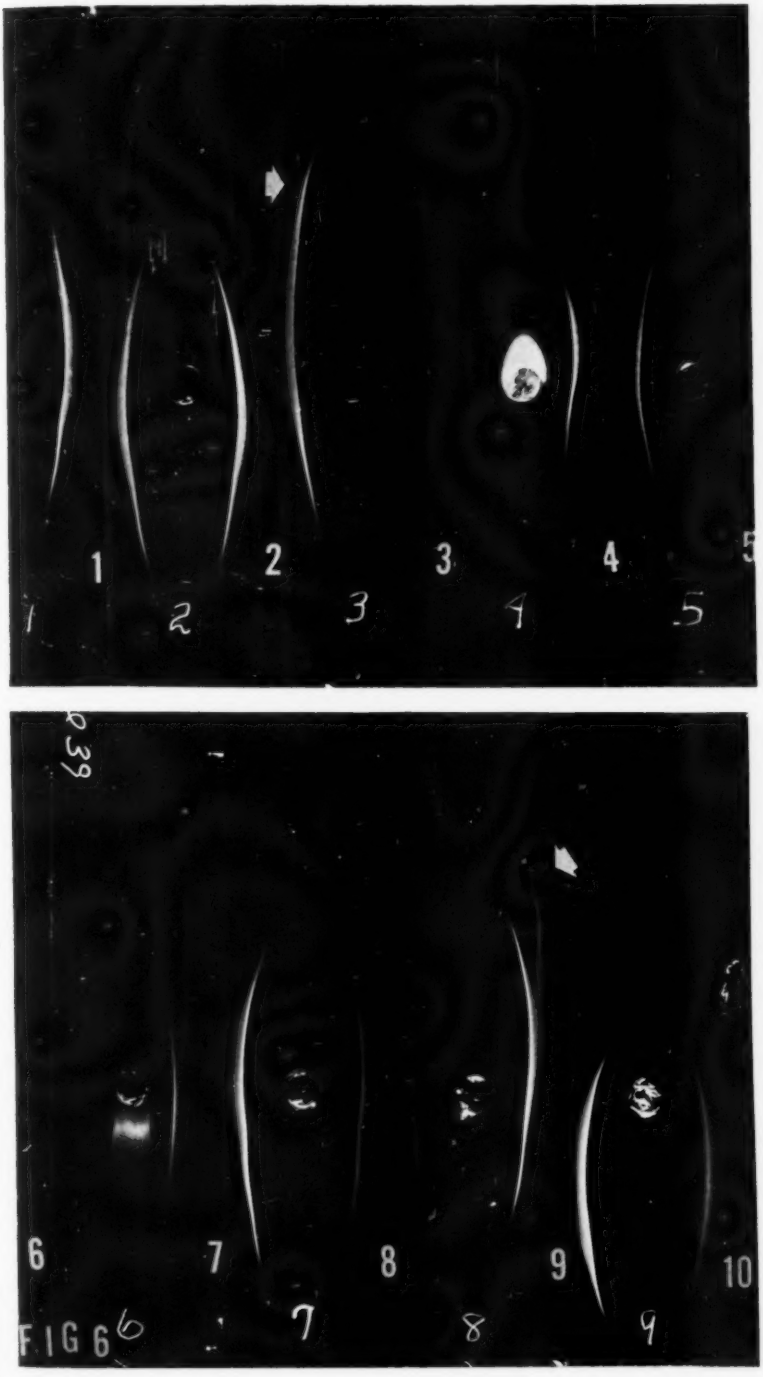


FIG 6

PLATE 152

FIGURE 7.—Immunoelectrophoresis to show relative mobilities. MPC-1 serum from transfer generations 8 (w 4) and 39 (w 2) were compared with MPC-4 (w 3) and Adj-PC-12 (w 1). *Note* similarities in shape and mobility of SII arcs formed in MPC-1 serum. *Note* dissimilarity of Ajd-PC-12 and MPC-4 when compared with MPC-1. MPC-2 Pw antiserum used throughout.

FIGURE 8.—Immunoelectrophoresis to show relative mobilities of MPC-2 (w 6) and MPC-3 (w 5); MPC-1 (w 3) and MPC-4 (w 4). Two fresh, unfrozen normal sera (w 1 and 2) were electrophoresed. MPC-2 Pw antiserum used throughout.

FIGURE 9.—Immunoelectrophoresis showing comparison of precipitating abilities of R1 antiserum (anti-MPC-3) before (t 2) and after absorption (t 1 and 3) with normal BALB/c serum. Normal BALB/c serum (w 1 and 4), MPC-2 serum (w 3), and MPC-3 serum (w 2) were electrophoresed. *Note* absorbed serum formed no reaction with normal serum. Long precipitin line beginning near 2 center wells and running extent of plate cathodally resulted from a precipitation of excess normal BALB/c serum antigens (accumulated during the absorption) and R1 serum.

FIG 7

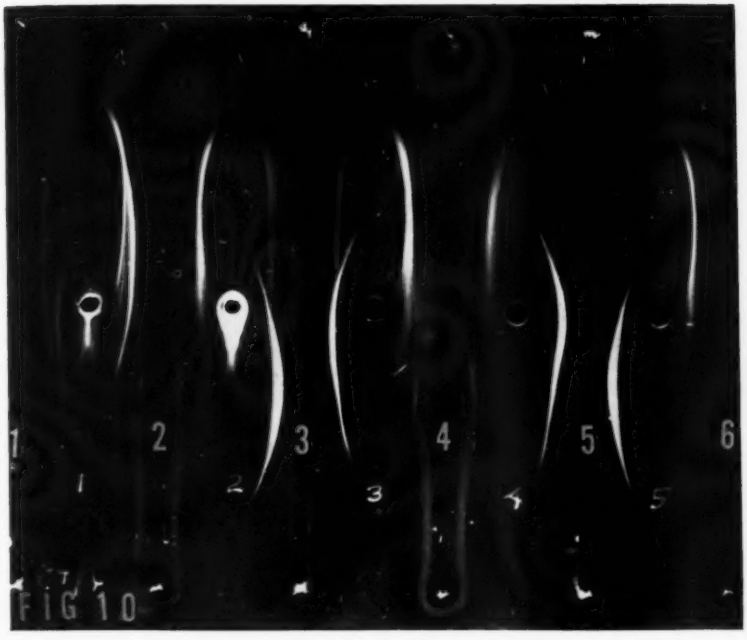
FIG 8

FIG 9

PLATE 153

FIGURE 10.—Comparison of MPC-2 Pw antiserum (t 1, 3, and 5) with Adj-PC-5 Pw antiserum (t 2, 4, and 6) for normal serum (w 1) and serum from mice bearing the different plasma-cell neoplasms Adj-PC-12 (w 2), Adj-PC-8 (w 3), MPC-3 (w 4), and MPC-1 (w 5). *Note* SII myeloma globulin arcs are not precipitated by Adj-PC-5 Pw antiserum.

FIGURE 11.—Difference between 2 transplant lines of Adj-PC-6 and normal serum (w 1), Adj-PC-6C (w 2), Adj-PC-6A (w 3). MPC-2 Pw antiserum used in all trenches. *Arrow* points to cathodal tip of Adj-PC-6C arc which fuses with inner SI arc. Contrast this pattern with Adj-PC-7 pattern (fig. 6, w 3) developed with the same antiserum where SII arc crossed both SI arcs.


1
2
3
4
5
6
FIG 10

1
2
3
FIG 11

PLATE 154

Ouchterlony Plates

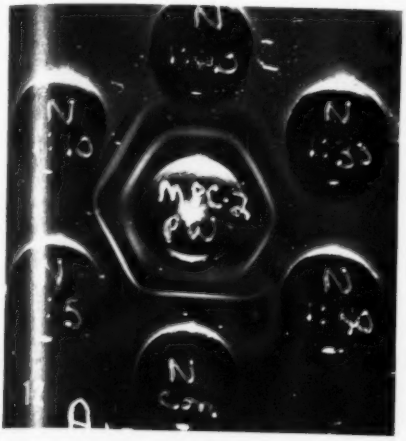
FIGURES 12 and 13.—Successive dilutions of normal BALB/c serum from whole serum in well labeled "N con" to a dilution of 1:100. *Note* disappearance of outer line after dilution of 1:10 and persistence of inner line through dilutions of 1:100. MPC-2 Pw serum in center well.

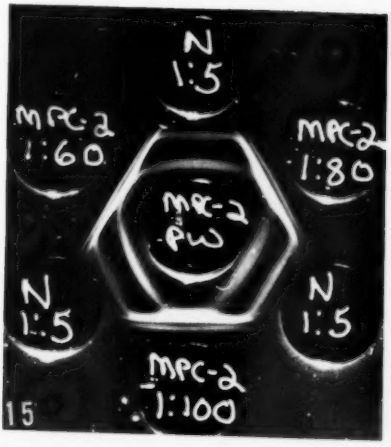
FIGURE 14.—Inner well MPC-2 Pw antiserum. Three wells at 2, 6, and 10 o'clock. Normal serum diluted 1:40. Three wells at 8, 12, and 4 o'clock contained successive dilutions of Adj-PC-5 serum, beginning with the 1:140 at 8 o'clock. At these high dilutions of Adj-PC-5, similarity to inner line was demonstrated. A new line close to the well appears.

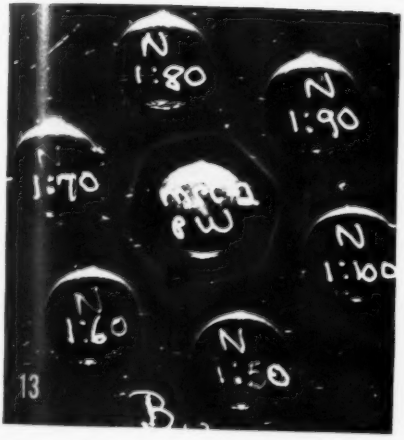
FIGURE 15.—Inner well contains MPC-2 Pw antiserum. Three wells at 4, 8, and 12 o'clock contain normal serum diluted 1:5. Two major lines develop at this dilution. In three wells at 10, 2, and 6 o'clock are successive dilutions of MPC-2. *Note* appearance of extra line developed in MPC-2 serum which runs into wells containing normal serum.

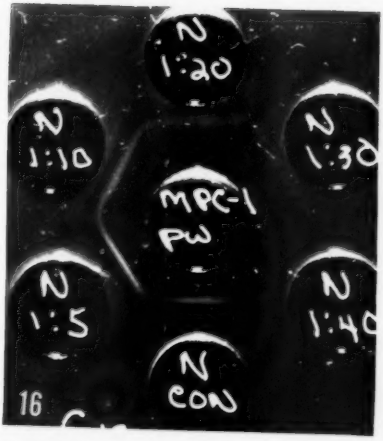
FIGURE 16.—Successive dilutions of normal serum from undiluted (N con) to 1:40. MPC-1 Pw in center well. Inner line is poorly developed. Outer line behaves like outer line developed by MPC-2 Pw serum (fig. 12) during successive dilutions.

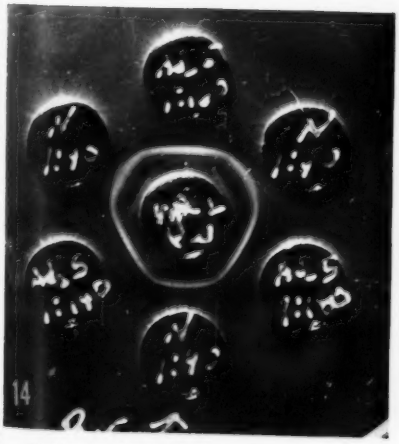
FIGURE 17.—Inner well contains MPC-1 Pw. Three wells at 2, 6, and 10 o'clock contain normal serum diluted 1:5. Three wells at 9, 12, and 4 o'clock contain successive dilutions of MPC-1 (as labeled). *Note* appearance of extra line developing on antigen side of outer line at dilutions 1:80 and 1:100 but not at dilution 1:60 of MPC-1.


N
1:10
N
1:50
MPC-2
PW
N
1:5
N
1:80
N
CON


N
1:5
MPC-2
1:60
MPC-2
1:80
MPC-2
PW
N
1:5
N
1:5
MPC-2
1:100
15


N
1:80
N
1:90
1:70
MPC-2
PW
N
1:100
N
1:60
N
1:50
13


N
1:20
N
1:10
N
1:30
MPC-1
PW
N
1:5
N
1:40
N
CON
16


14

MPC-1
1:80
N
1:5
N
1:5
MPC-1
1:60
MPC-1
1:100
N
1:5
17

Sarcomatous Change and Maintenance of Differentiation in Long-Term Cultures of Mouse Mammary Carcinoma [1]

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SUMMARY

This study had 2 objectives: 1) to follow the behavior *in vitro* of cells cultured from C3H mouse mammary carcinomas with respect to maintenance of differentiation as assayed *in vivo* and 2) to supply additional evidence regarding the "sarcomatous" transformation which epithelial tumors frequently undergo and which appeared in cell strains of this study. Cell strains were established in culture from 18 different tumors of mammary-gland origin. Cells cultured in a serum-embryo extract medium and transferred from culture to mice during the first 5 months *in vitro* grew as differentiated mammary carcinomas, but cells transferred after this period produced sarcoma-like tumors. Experimental evidence indicated that the "sarcomatous" changes in the various cell strains resulted from two entirely different phenomena: 1) neoplastic transformation of stromal cells in culture, and 2) morphologic alteration of carcinoma cells to resemble sarcoma cells of connective-tissue origin. One strain cultured from the long-transplanted C3HBA mammary carcinoma and grown in serum-supplemented chemically defined medium continued to reproduce in mice the epithelial glandular architecture of the original tumor after 1½ years and 35 transplant generations of growth *in vitro*. The high arginase activity characteristic of C3H mammary carcinomas was also maintained. Apparently two factors accounted for the maintenance of recognizable differentiation in cells of this strain: 1) the type of culture medium used, and 2) the stabilization of tumor morphology and differentiation during numerous passages *in vivo* before explantation of tumor tissue to culture.—J. Nat. Cancer Inst. 26: 1139-1183, 1961.

THIS PAPER presents some observations on long-term cell strains cultured *in vitro* from spontaneous mammary tumors, both primary and transplanted, taken from strain C3H mice. At intervals during growth *in vitro*, we reimplanted cultures into mice to determine whether the cells after prolonged culture could reproduce *in vivo* the specific morphologic characteristics of the tumor from which they were obtained. The regular production of sarcoma-like tumors from some of these implants

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[2] Laboratory of Pathology.
[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

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raised certain questions which led to the experiments reported in the second section.

The second section describes and summarizes the results of clonal analyses and transplantation experiments carried out on 5 cell strains to elucidate the origin of the sarcomatous tumors. Two hypotheses were tested: 1) The tumors might arise from stromal cells that had become malignant during culture, or 2) the tumors might arise from the primary carcinoma cells that had undergone a hereditary alteration during prolonged culture to resemble fusiform cells of a fibrosarcoma.

MATERIALS AND METHODS

Source of cells.—Cell strains were established in culture from 18 different tumors of mammary-gland origin of which 9 were primary tumors taken from strain C3H female mice. Three were transplanted tumors carried for 2 generations in reciprocal F_1 hybrids between mice of strains C3H, containing the mammary-tumor agent, and BALB/c (referred to hereafter as C) without the agent. Six were transplants of the mammary adenocarcinoma, C3HBA, which originated in a strain C3H mouse (*1*).[4] Four of the 6 transplants had been carried for 118 to 129 generations in strain C3H, and 2 had been carried for 33 generations in strain C3H, 7 generations in hybrids, (C3H × C)F_1, and 146 and 153 generations, respectively, in backcross animals (C3H × C)CBC. Sections of each tumor were fixed in Zenker-formol and stained with hematoxylin and eosin and with van Gieson's picrofuchsin. From examination of these sections, we diagnosed all the tumors used for tissue culture, with one exception, as adenocarcinomas, types A or B as classified by Dunn (*2*). The exception, to be described later, was diagnosed as a sarcoma and was a second-generation transplant of an adenocarcinoma type B. Type A adenocarcinomas are typical mammary tumors of basic acinar structure, whereas type B tumors show a variety of epithelial patterns and often contain cysts filled with blood or clear fluid. Intracystic papillary projections, cords, and tubes of cells also characterize type B tumors. Both tumor types are clearly of glandular epithelial origin and are frequently associated with the presence of the milk agent.

Culture methods.—The tumor was dissected aseptically from the mouse and cut into strips, each about 12 × 6 × 2 mm. These strip-shaped explants were either embedded in hen-plasma clot or inserted under a sheet of perforated cellophane in a D-3.5 Carrel flask, according to procedures described (*3*). Because the cells tended to lyse the plasma clot, a sheet of perforated cellophane was often lowered onto the clotting mixture to provide a second substrate for cell growth. After the first-transplant generation, cells of some strains became adapted to grow on the glass floor of the flask and were carried thereafter without substrate

[4] These tumors, as well as certain of the F_1 hybrid mice referred to later, were kindly supplied by Dr. Morris K. Barrett of the Biology Laboratory of this Institute.

of plasma clot or cellophane. Other cell strains were continued in plasma clot or under cellophane.

Two types of fluid culture medium were used. The first, which will be referred to as "stock medium," consisted of a filtered mixture of 40 percent horse serum, 20 percent chick-embryo extract (1:1.25) and 40 percent Earle's saline. The second, referred to as "experimental medium," consisted of chemically defined medium NCTC 109 supplemented with 10 percent horse serum (*4*, *5*). No antibiotics were used in any of the culture media because of the complications their usage might introduce. The fluid medium was renewed 3 times a week at which time the cultures were gassed with 5 percent CO_2 in air to adjust the *p*H to approximately 7.3.

After the first few transplant generations in culture, the cells grew rapidly and were transferred to 2 or 3 new flasks approximately every 2 weeks. Often the parental culture was also maintained and allowed to repopulate. When larger quantities of cells were needed for injections, 3 or 4 cultures in Carrel D-3.5 flasks were pooled to inoculate one T-60 flask (*6*).

Of the 18 cell strains, 13 were cultured in stock medium. Four strains and all single-cell clones were cultured in experimental medium. Strain 2291 was started in stock medium, and after 20 weeks was transferred to experimental medium. Occasionally, we transferred individual cultures grown in one type of medium to the other type to determine whether the culture medium influenced cell morphology. No such effect was observed.

Photographic procedures.—Photographs of the living cells were made by procedures described (*7*). Cells were photographed 24 hours after a renewal of culture fluid.

Cloning procedure.—Six single-cell clones and several culture lines originating from 2 or 3 cells were established by a modification of the cloning procedure originally described (*8*). Sieved suspensions of cells were prepared in experimental medium. The cell suspensions were drawn freehand without microscopic observation into capillary pipettes that were sealed, examined, and cut into segments containing single cells. Each capillary segment was then transferred to a separate Carrel flask, the floor of which was coated with a thin film of plasma-clotting mixture to anchor the capillary. We made microscopic observations to verify the presence of one cell in the capillary of each flask. The progeny of the single cell ultimately grew out both open ends of the capillary to fill the flask.

Procedures for inoculation of tissue-culture cells and tumor tissue into mice.—The procedures for inoculation of culture cells and tumor tissue into thigh muscles of mice have been described (*9*). In a few of the earlier experiments, cells were injected subcutaneously into the right or left axillary regions rather than into the thigh muscles. Mice used for inoculation were males, 1 to 6 months of age, mainly of strain C3H/An. However, some mice of strains C3Hf/He, C3H/HeN, and reciprocal F_1

hybrids between mice of strains C3H/HeN and C(BALB/cAnN) were also used. Certain mice, as indicated later, were irradiated with a whole-body dose of 425 r several hours before inoculation to lower their resistance.[5] Physical factors were 200 kv, 15 ma, 120 r per minute at a focal distance of 54 cm through 0.25 mm Cu and 0.55 mm Al filters.

Mice were kept under observation for at least 3 months after inoculation. The site of inoculation was palpated weekly to determine the latent period for tumor development. The latent period measured the interval from injection of cells to the appearance of a palpable tumor at the site of injection. Autopsies were carried out on all mice, and sections of tumor tissue were fixed and stained as described.

EXPERIMENTS AND RESULTS

I. Observations on Cell Strains

Morphology In Vitro

During the 1st few weeks of culture, tongues and sheets of epithelial cells migrated from the tumor explants (figs. 1, 4, and 8). These membranous sheets ultimately broke up into individual irregular-shaped cells. The disruption of cell sheets was particularly noted in lysed areas of the plasma clot (figs. 2 and 3) and in the cellophane-substrate cultures. Some cultures also contained elongated, fibroblast-like cells that were considered to be either fibroblasts from the stroma of the tumor or possibly modified tumor cells (fig. 5).

After 6 to 12 months of proliferation in culture, the morphology of the cell strains stabilized. Some strains in each type of medium were epithelial in morphology, growing as flattened cells in membranous sheets (figs. 6, 21, and 22); others were fibroblast-like, forming networks of elongated, spindle-shaped cells (figs. 16, 17, and 20); and still other strains were difficult to classify either because they were morphologically intermediate between the epithelial and fibroblastic type and showed greater variation in cell structure or because they were obviously mixed (figs. 9, 18, and 19). Cultures of strain 2777 frequently contained islands of epithelial cells separated by networks of fibroblastic cells. Two single-cell clones (3025 and 3026) isolated from this strain were epithelial in morphology, whereas one subline from 3 isolated cells (subline 2976, table 1) was distinctly fibroblastic (fig. 20).

Behavior In Vivo *and Morphology of Tumors*

After different periods of growth *in vitro*, we reimplanted cells into mice to determine whether after prolonged culture the cells could reproduce *in vivo* the morphologic characteristics of the tumor from which

[5] The authors express their appreciation to Mr. Henry Meyer, of the Radiation Branch of this Institute, who irradiated the mice.

they originated. Cultures from 11 of the 18 cell strains and 5 of the derived clones were injected into mice. Mice of strain C3H as well as reciprocal F_1 hybrids between C3H and C were used. A different culture was injected into each mouse. The results of these injections are summarized in table 1 which compares the tumors produced by the injected cultures with the tumor from which the cell strain originated.

TABLE 1.—Comparison of tumors from which culture cell strains originated with tumors produced by the cells after different periods of culture *in vitro*

Cell strain, clone, or subline	Tumor from which cell strain originated*	Weeks in culture	Results of injections†	Individual tumor latent periods‡ (weeks)	Tumor produced by injected cultures*
1333§	A	10–20	3/7	9,14(2)	A(2),B
1335§	B	10–20	2/6	6,11	B(2)
1348§	A	7	1/2	13	B‖
		11–26	0/14	—	—
		40–288	5/12,2/2‖	7,9,10(3) 11,13	S(6)
Clones:					
2442		24–46	3/3,3/3‖	1(5),2	A.C.(6)
2443		13–46	2/2,2/5‖	1(4)	S, A.C.(3)
2444		13–46	2/3,3/4‖	1(4),2	A.C.(4)
1349§	B	7	1/2	13	B
		11–26	3/4	8,10,17	S(3)
1351§	B	7–17	2/5	19,52	B(2)
1352§	A	11–26	0/9	—	—
2291	—	41–65	7/7‖	3(6)	S(5)
2577	S	26	3/3	2 (3)	S(3)
2848	A	33–58	4/4,1/2‖	2(4), 3	S(5)
Clone: 2978		25	1/1‖	2	S
2940	A	21–49	1/3‖	11	B
2777	B	11–40	2/11, 10/13‖	2(10),4(2)	B(10)
		50	1/3‖	11	S & B mixed
		65–82	10/10‖	5(3),6,7(5) 9	B(10)
Clone: 3025					
Subline 2976		25	0/1‖	—	—
		25–57	7/8‖	5(4),7(2)8	S (7)

*A = mammary tumor, type A, of basic acinar structure; B = mammary tumor, type B, of variable epithelial glandular pattern; S = fusiform-cell tumor resembling fibrosarcoma; A.C. = tumor resembling anaplastic carcinoma. Number of mice bearing tumors of each type is indicated in parentheses.

†Number of mice with tumors divided by number of mice receiving injections. Each mouse received a different culture.

‡The tumor latent period measured the interval from injection of cells to the appearance of a palpable tumor at site of injection. Number in parentheses indicates number of mice developing tumors of the latent period specified.

§Cells cultured in stock medium. All other cells cultured in experimental medium.

‖Mice irradiated before injection.

During the first 21 weeks of culture, cells reimplanted into mice grew as differentiated mammary carcinomas of types A (fig. 23) or B. Of the 8 strains continued for more than 21 weeks *in vitro*, 5 produced tumors composed of fusiform cells resembling sarcomas (fig. 24). Strains 1352 and 2940 failed to grow in mice, and strain 2777 continued to produce numerous type B adenocarcinomas like the tumor of origin. Even after 1½ years and 35 transplant generations of growth in experimental medium, cells of this strain reproduced in mice the glandular structure of the original type B tumor. Some of the resulting tumors, however, contained areas of pale, undifferentiated cells resembling anaplastic carcinoma, or other areas composed of fusiform cells. Only one section was found without some glandular areas. This suggests that these tumors were in a transitional state (figs. 25 and 26).

The sarcoma-like tumors appeared after latent periods averaging 11 weeks for cells grown in stock medium and 4 weeks for cells grown in experimental medium. Thus, the type of culture medium significantly influenced the latent period for tumor development, as has been shown for other tissue-culture cell strains (*10*).

One fusiform-cell tumor produced by injecting a culture of strain 1348 cells into a mouse was explanted to culture. The cells in culture showed a more fibroblast-like morphology than cells of the strain not grown in mice (figs. 6 and 7).

In summary, cells transferred from culture to mice during the first 21 weeks of growth *in vitro* grew as differentiated mammary carcinomas of types A or B. Cells transferred after this period of culture, with few exceptions, produced sarcoma-like tumors.

II. Experiments Concerning Production of Sarcoma-Like Tumors by Injected Cultures

The object of these experiments was to elucidate, if possible, the origin of the fusiform-cell tumors produced by the injected cultures. Four possibilities were considered. The tumors might be induced in the host tissues by the cultures, either the cells or the culture fluids. For several reasons this possibility seemed unlikely. No sarcomas were produced during the first 21 weeks in culture when the carcinoma cells were often successfully transplanted and were shown to carry the mammary-tumor agent (*11*). Also, the latent periods for development of fusiform-cell tumors in mice were relatively short for an induction phenomenon unless an exceedingly active carcinogenic agent was present in culture. Finally, no sarcomas developed in the many young or adult mice of C3H or homologous strains used for assaying the culture fluids or cells for the mammary-tumor agent (*11*).

A second possibility was that the original tumors were mixed or "carcinosarcomas." However, histologic examination of the primary tumors and later transplants *in vivo*, with possibly one exception to be described later, furnished no evidence for such a mixture of cell types.

A third possibility was that, in the cultures, stromal cells from the original tumor explant might overgrow the primary tumor cells, undergo a neoplastic change, and grow into sarcomas when the cultures were reinoculated into mice. The fusiform-cell tumors would thus be true sarcomas originating from the stromal cells in culture.

A fourth possibility was that the carcinoma cells of epithelial origin might undergo a morphologic change during culture to resemble sarcoma cells of mesenchymal origin. The fusiform-cell tumors would thus be morphologically altered carcinomas. The latter two possibilities were investigated in the following experiments carried out on five cell strains.

Experiments on Cell Strain 1348

Cell strain 1348 was derived from a spontaneous mammary carcinoma of type A (table 1). When returned to mice after 7 weeks of culture in stock medium (3 transplant generations), the cells produced a differentiated adenocarcinoma (type B), not significantly different from the tumor of origin. After 40 weeks of culture, the cells produced tumors composed of fusiform cells resembling fibrosarcomas. These tumors developed slowly after latent periods of 7 to 13 weeks. Examination of the cells in culture at this time revealed almost pure epithelial sheets (fig. 6). From this strain, 3 single-cell clones were developed. In the establishment of these clones, it was necessary to grow the single cells in experimental medium rather than in the stock medium used for the parental strain.

Experimental results.—Each of the three clones (2442, 2443, and 2444, table 1) resembled the parental strain in showing an epithelial morphology when cells were cultured in plasma clot or on the glass floor of the flask (figs. 10, 11, 14, and 15). However, in clone 2443 some areas of elongated fibroblast-like cells were seen (figs. 12 and 13). When injected into mice, cells from each of the clones produced tumors that differed in three ways from tumors produced by the parental strain: 1) The latent period for tumor development ranged from 1 to 2 weeks as compared with 7 to 13 weeks for cells of the parental strain (table 1). 2) On gross examination, the tumors were cystic rather than solid and were filled with clear viscous or bloody fluid. Often the thick cyst wall had papillary extensions into the cavity. 3) On histologic examination, areas of tumor tissue within the walls of the cyst resembled such undifferentiated tumors, or anaplastic carcinomas, as Sarcoma 37 or Sarcoma 180, both of which originated as mammary tumors. The cells were epithelial-like, round, or oval; the cytoplasm was scanty, slightly eosinophilic, and homogeneous; and the cell outlines were indistinct, so that areas of the tumor appeared syncytial. Mitotic figures were abundant (fig. 27). In some tumors there was a marked variation in the degree of basophilia in different areas of the tumor (fig. 28). In one tumor produced by cells of clone 2443, some areas resembled anaplastic carcinoma, while other areas were composed of fusiform cells (fig. 29, A and B). This tumor, as well as all other tumors from

the three clones, resembled anaplastic carcinoma after passage for one or more additional generations in mice (table 2).

Comments.—There are several possible interpretations of the different behavior *in vivo* of the clones as compared with the parental strain. The different culture fluid used for the clones may not only have enhanced their transplantability but also may have preserved their differentiated epithelial character during growth *in vivo*. The anaplastic carcinoma-like architecture of tumors produced by the clones as well as the sarcoma-like

TABLE 2.—Results of transplanting tumors produced by injected cultures

Cell strain, clone, or subline	Mice used for transfer	Number of tumors sub-inoculated	Maximum number of generations *in vivo*	Results of inoculation*	Tumor developing from transplant†
1348	C3H	2	5	24/24	S(8)
Clones:					
2442	C3H	1	2	0/3	—
	(C × C3H)F_1	2	5	6/10	A.C.(6)
2443	C3H	3	3	14/17	A.C.(7)
	(C × C3H)F_1	1	6	12/13	A.C.(10)
2444	C3H	1	2	1/3	—
	(C × C3H)F_1	1	2	0/3	—
2291	C3H	1	2	3/3	S
2577	C3H	3	2	6/6	S(4)
	(C × C3H)F_1	3	2	6/6	S(3)
2848	C3H	3	2	0/14	—
	(C3H × C)F_1	1	2	5/5	S(5)
	C	1	2	0/3	—
Clone:					
2978	C3H	1	2	0/5	—
	(C3H × C)F_1	1	2	5/5	S(5)
2777	C3H	7	2	14/15	B(9)
	(C3H × C)F_1	4	2	11/11	B(10)
	C	1	2	0/4‡	—
	C3H*f*	1	4	14/14	S(7)S + B(2)
	(C × C3H)F_1	1	2	1/1	S
Subline:					
2976	C3H	7	2	0/9	—
	(C3H × C)F_1	7	2	8/8	S(8)

*Number of mice with tumors divided by number of mice inoculated.

†S = fusiform-cell tumor resembling fibrosarcoma; A.C. = tumor resembling anaplastic carcinoma; B = mammary tumor, type B, of variable epithelial glandular pattern. Number of mice bearing tumors of each type is indicated in parentheses.

‡Seven of the 14 mice were irradiated before inoculation.

character of tumors produced by the parental strain persisted during numerous transplants *in vivo* (table 2). If the difference in morphology is attributed to the different culture media used, the media may have induced some hereditary change in the cells to account for persistence of the morphologic difference during generations of growth in mice.

A second less likely interpretation is that the tumors produced by the clones, after short latent periods, arose from the implanted cells, whereas those produced by the parental strain, after longer latent periods, originated from host tissues.

Another interpretation is that the cell population of the clones may have differed from that of the parental strain. Stromal cells, which could have become malignant during long-term culture (*9*), may have been present in the parental strain, but absent from the single-cell clones.

The morphology of the tumors *in vivo* was particularly interesting. Growth of tumor cells around a fluid-filled cyst has not been observed before, after the injection of cells from tissue culture. However, cyst formation is characteristic of many mammary carcinomas. The variable morphology of the cells comprising the tumors indicates that the descendants of a single cell may display widely divergent morphologic characters.

Cells of the three clones showed no activity of the mammary-tumor agent, which was detected in the parental strain during the first 7 weeks of culture (*11*).

The clones also showed no activity of the enzyme arginase as reported by Westfall and associates (*12*). An unusually high arginase activity has been found in differentiated mammary carcinomas of the strain C3H mouse (*13*). In these two respects, the clones differed from morphologically differentiated mammary carcinomas of strain C3H mice.

Experiments on Cell Strain 2577

Several cell strains were established in culture from transplanted tumors. By inoculating small fragments of the tumor into compatible F_1 hybrids (C3H $\times$ C)F_1, allowing the tumor to reach a large size in the hybrid, and by taking only the outer periphery of the growth for further inoculations, we would presumably replace the C3H stroma of the primary tumor, after several such transfers, with stroma from the hybrid host (*14*).

All tumor transplants used for culture were such mixtures of C3H tumor cells and hybrid stromal cells. These two cell types could generally be distinguished, since cells of C3H origin would be transplantable to C3H and hybrid hosts, whereas cells of the hybrid would be transplantable to the hybrid but not to C3H hosts (*15, 16*).

Experimental results.—The tumor used to establish cell strain 2577 originated in a strain C3H female as a mammary adenocarcinoma, type B, and was carried in compatible hybrids for 8 generations. During this period, the tumor varied somewhat in the 47 mice inoculated, but generally maintained the type B histologic pattern, with one exception. In the second passage, a fusiform-cell tumor developed at the inoculum site. Because the histologic change was not recognized at the time, this tumor was used to originate cell strain 2577.

Cells of strain 2577, after 26 weeks of growth in culture, were implanted into 3 irradiated hybrid mice (C $\times$ C3H)F_1. Tumors composed of fusiform cells developed (table 1). Three of these tumors, each from a different culture, were subinoculated and grew progressively in both strain C3H and hybrid hosts (table 2). These observations seemed to identify the cells of strain 2577 as of strain C3H origin.

Comments.—In this experiment a sarcomatous change appeared to take place during transplantation of the C3H mammary carcinoma in hybrid

hosts, but the fusiform-cell tumor that developed in the hybrid was apparently not derived from host tissues but from the original C3H tumor. These observations suggest three possibilities: 1) Even though the sections taken from the primary tumor were diagnosed as type B mammary adenocarcinoma, the tumor may actually have been a mixed carcinosarcoma. This possibility seems unlikely, since only one sarcoma developed in the 47 mice inoculated, and sarcoma cells generally overgrow carcinoma cells in the transplantation of tumors. 2) The stromal cells of the original C3H tumor may have become neoplastic during the first or second generation of transfer in the compatible hybrid host. 3) Certain of the primary tumor cells may have undergone a hereditary change in shape during growth in the hybrid host and come to resemble the fusiform cells of a fibrosarcoma. These latter two possibilities will be discussed in more detail later.

Experiments on Cell Strains 2848 and 2940

Cells of these two strains were originated in culture from different transplants of the same tumor. The tumor arose spontaneously as a type B mammary adenocarcinoma in a strain C3H female mouse and was carried for two generations in compatible F_1 hybrids (C3H $\times$ C)F_1. The second-generation transplants used for culture were classified as type A.

Experimental results.—During the early weeks of culture, cells of both strains formed epithelial cell sheets. Later, elongated fibroblast-like cells appeared in strain 2848 and seemed to overgrow the cultures, whereas cell strain 2940 maintained the epithelial morphology during the subsequent years of growth (figs. 21 and 22). After 18 weeks *in vitro*, one cell was isolated from a culture of strain 2848 and was grown to clone 2978 (table 1). Cells of the clone and parental strain were almost indistinguishable morphologically (figs. 16 and 17), and, when transplanted to hybrid mice after 33 to 58 weeks of culture, produced sarcoma-like tumors (table 1).

These tumors were palpable as early as 2 weeks after cell implantation, and 4 were subinoculated and exhibited a specificity for the hybrid host, failing to grow in mice of strains C3H or C (table 2). These tests seemed to identify the cells of strain 2848 and of the clone as of hybrid origin.

Cells of strain 2940 cultured in the same type medium as cell strain 2848 from a transplant of the same tumor maintained the epithelial morphology and failed to grow when implanted into 2 irradiated mice after 49 weeks of culture (table 1). The cells from culture were assayed for the enzyme arginase according to procedures described (*12*), since differentiated mammary carcinomas of the strain C3H mouse reportedly exhibit a high activity of this enzyme (*13*). As may be noted (table 3), the cells showed arginase activity when assayed after 66 weeks of culture and 19 transplant generations of growth *in vitro*. Assays for the mammary-tumor agent furnished some evidence for persistence of the agent in these cells (*11*). In these two respects, cells of strain 2940 still resembled

TABLE 3.—Arginase activity of cultured cells

Cell strain, clone, subline, or tumor	Number of T-60 cultures used	Total wet weight of cells (mg)	Mg urea/minute/100 g protein
2940	1	20*	300
2777	2	47	3200
Tumor from 2777	—	—	7200
3025	1	7*	310
2976	2	54	0
	3	132	0
2071	3	93	0

*We have had no experience in grinding quantities such as these in which the volume had to be greatly reduced over that used in our standardized procedure (*18*). The arginase extraction may be poor from these two samples.

mammary-carcinoma cells and differed from the three single-cell clones described earlier from strain cell 1348.

Comments.—Two types of cell appeared to have been isolated in culture from the original tumor. One type resembled the primary carcinoma cell, though limited efforts to transplant the cells into mice were unsuccessful. The second type apparently originated from the stroma of the hybrid host, overgrew the primary tumor cells in culture, became malignant by 8 months *in vitro*, and gave rise to fusiform-cell sarcomas when transplanted to mice.

Experiments on Cell Strain 2777

Cell strain 2777 was derived from a transplant of Barrett's C3HBA mammary carcinoma that arose spontaneously in a strain C3H female and had been carried for 186 generations *in vivo*, the last 153 generations in (C3H × C)F_1 and (C3H × C)CBC hosts. This tumor has never been known to undergo sarcomatous change during the years of transfer in mice.

Cells of strain 2777 in culture showed from the first a diffuse necrosis that so far has not been linked to any detectable bacterial or virus contamination, though the mammary-tumor agent probably persisted in the cells (*11*).

Experimental results.—After 25 weeks of culture, cells were isolated to establish 2 single-cell clones (3025 and 3026), and a subline (2976) developed from 3 cells (table 1). Cells of the 2 clones proliferated slowly as epithelial sheets showing some diffuse necrosis. Cells of ths subline grew rapidly and appeared fibroblastic. In the parental strain both fibroblast-like and epithelial cells persisted for the 2½ years of culture. The epithelial cells were often surrounded by networks of fibroblastic cells.

Numerous cultures of cell strain 2777 were transplanted to C3H mice or to reciprocal hybrids between C3H and C. All tumors developing from the injections were mammary carcinomas of type B similar to the tumor from which the culture strain was derived, with one exception (table 1). The exception was a sarcoma-like tumor containing carcino-

matous areas (fig. 26) that appeared in an agent-free C3Hf mouse after a latent period of 11 months. The mixed tumor was transplantable to nonirradiated C3H hosts and was carried for 4 generations in C3H mice (table 2). The cells thus appeared to be of C3H origin.

The cells of strain 2777 showed an exceedingly high activity of the enzyme arginase, characteristic of differentiated mammary carcinomas of the mouse (table 3). A tumor produced *in vivo* by implantation of the cells exhibited an even higher activity. The higher activity in the tumor than in the cells from culture may have resulted from concentration of the arginase-producing cells or from increased synthesis of this enzyme in the mouse.

No tumor was obtained from the epithelial-like clone 3025 which also showed arginase activity (table 3).

Cultures of subline 2976 were injected into hybrid mice (C3H $\times$ C)F_1 and produced sarcoma-like tumors that were transplantable to the hybrid but not to C3H mice (tables 1 and 2). These observations served to identify cells of this subline as from the hybrid host.

When assayed for arginase activity, cells of subline 2976 exhibited no activity (table 3). For comparison, a culture strain of C3H mouse cells originating from normal connective tissue (strain 2071 from strain L) was tested and likewise showed no arginase activity.

Comments.—At least three different cell types appear to have been isolated from strain 2777. One was the primary tumor cell which, when transplanted and carried for several generations in strain C3H mice (table 2), reproduced in morphologic pattern the type B mammary adenocarcinoma from which the cells were originated in culture. These cells had a high arginase activity and possibly carried some mammary-tumor agent.

The second cell type, isolated in subline 2976, was the stromal fibroblast, which apparently during culture became malignant, growing into sarcomas when implanted into hybrid mice. The isolation and identification of this cell type further confirmed the concept set forth earlier that cells of the stroma of the tumor may become malignant in tissue culture and give rise to sarcomas when implanted into mice.

The third cell type was a sarcoma-like cell transplantable to C3H mice and, therefore, presumably of C3H origin. These cells produced by injecting a culture of strain 2777 into a C3Hf mouse possibly arose from host tissues. However, in view of the exceedingly rare occurrence of spontaneous sarcomas in young mice of this strain (*17*) and the inability to demonstrate the induction of sarcomas with the cells or culture fluids of strain 2777, it seems more likely that the sarcoma arose from the implanted cells. If so, one or more of the carcinoma cells from culture would appear to have undergone a hereditary morphologic change to give rise to the sarcoma-like tumor. The tumor used to initiate cell strain 2777 had been carried for years (153 serial transfers) in hybrid or backcross animals, and had never been observed to undergo sarcomatous change. After this period of transfer, the occurrence in the transplant of any C3H stromal cells from the original tumor would be most improbable. There-

fore, any sarcoma-like tumor of C3H origin, developing from the implanted cells, would appear to have originated from the carcinoma cells in culture.

DISCUSSION

Analysis of the "sarcomatous" changes *in vitro* in different cell strains of this study supplies additional evidence regarding the "sarcomatous" transformation that epithelial tumors frequently undergo during transplantation *in vivo*. This phenomenon was first reported in 1905 by Ehrlich and Apolant (*18*) for a spontaneous mammary adenocarcinoma of the mouse serially propagated *in vivo*. At the 10th generation of transplantation the growth at the site of inoculation contained both carcinomatous and sarcomatous areas, and by the 14th generation the tumor appeared to be a pure sarcoma and remained so thereafter. Two other such changes in mouse mammary carcinomas (after the 16th and 68th generations *in vivo*), were also described by these workers (*19*). Because each of these 3 tumors was propagated over a prolonged period and through numerous generations as a carcinoma, with no histologic evidence of sarcoma, the authors felt convinced that the primary tumors were not mixed (*20*). They favored the interpretation that, in the course of continued propagation, the chemical metabolism of the carcinoma cell became so altered that substances were formed which had a stimulatory action on the connective-tissue scaffolding of the tumor. The carcinoma cells thus induced a sarcomatous change in the connective tissue of the host.

Lewin (*21*) reported that during 11 transplantations of a mammary adenocarcinoma of the rat, adenocarcinomas, spindle-cell sarcomas, round-cell sarcomas, and mixtures were obtained. Again, there was no histologic evidence that the primary tumor was mixed. Two possible explanations for the "sarcoma" development were suggested. The carcinoma cells may have transformed into spindle cells resembling sarcoma or the host connective-tissue cells may have undergone sarcomatous change as postulated by Ehrlich and Apolant.

During transplantation of a slow-growing adenocarcinoma with somewhat fibrous stroma, Haaland (*22*) observed the development of what appeared to be sarcomatous interstitial tissue in several separate strains. This tissue outgrew the carcinoma elements in subsequent generations and gave rise to pure "sarcoma" or occasionally the tumor reverted to carcinoma. Characteristic fine fibrils in the spindle-shaped cells and other fibrils outside the cells giving the reactions of collagen convinced Haaland that the growths were true sarcomas of connective-tissue origin. Although he recognized that carcinoma cells, particularly at the periphery of alveoli, can assume spindle-shape, he found no evidence for transformation of carcinoma elements into sarcoma. The possibility of an initially mixed tumor was excluded, as before, from histologic examination of the primary tumor and the early transplants and because of the long period

which elapsed before the change occurred. The observations suggested a possible connection between sarcoma development, low growth energy of the carcinoma, and enhanced resistance of the host. Two groups of conditions were considered important for sarcoma development: 1) repeated transplantation of the stroma which he felt might survive during transfer into successive hosts, and 2) formative influences exerted by the carcinoma cells.

Russell (*23*) described "sarcoma" development during propagation of a hemorrhagic mammary adenocarcinoma of the mouse. He believed that if a carcinoma is allowed to remain for a long period, at least 60 days, of sustained growth in one animal, a type of stroma is obtained which survives transplantation, has a high proliferative power, and is endowed with properties of a malignant new growth or sarcoma. According to Russell, the carcinoma cells are responsible for induction of malignancy in the stromal cells.

Bashford (*24*) described two types of change in mouse mammary carcinomas, one in which the parenchymal cells assume spindle-form to resemble sarcoma histologically but often revert to adenomatous character in older tumors, and one in which the carcinoma cells induce a sarcomatous change in the connective-tissue stroma. Four tumor strains appeared to undergo the latter change, two as primary tumors and two during transplantation. One of the latter, #37, was carried as two separate substrains. In one substrain the first type of polymorphism in parenchymal cells occurred, but the carcinomatous character was restored during prolonged transplantation. In the other substrain, a sarcomatous change in the stroma appeared to take place 4 times during serial propagation, which frequently led through the mixed-tumor stage to pure "sarcoma." Bashford also concluded that a tumor, rapidly propagated so as to remain less than 5 weeks in one animal, tended to remain carcinomatous, whereas sarcomatous change tended to supervene in slow-growing tumors grown progressively for more than 8 weeks in one host. However, once initiated, the sarcoma usually rapidly overgrew the carcinoma cells. Bashford raised a question as to whether the power to induce sarcoma development is a transitory attribute of certain carcinomatous parenchyma or a passing phase in some and permanent property in others.

In 1916 Ewing (*25*) raised doubts concerning the generally accepted conclusion of these earlier investigators that certain mouse tumors can produce a sarcomatous transformation in their stroma. Since spindle-shaped tumor cells are common in carcinomas, especially in those from human beings, their occurrence in any cancer, according to Ewing, is very strong presumptive evidence that they are altered epithelium. The sudden change in morphology and increased growth rate of the tumor might result from a morphologic alteration of epithelial cells into spindle cells, a more resistant and more rapidly growing form of epithelial cells. Certain difficulties in interpreting transitional cell types from histologic sections were pointed out. "In view of all these difficulties," wrote

Ewing, "it may be urged that further evidence is required before the sarcomatous transformation of mouse carcinoma can be accepted as proven. The writer would not deny its occurrence but merely asks for more evidence."

Two years later Woglom (*26*) wrote: "That there does occur in the mouse a type of new growth in which the epithelium assumes a spindle shape can scarcely be denied. Still, this may not be the whole story." In second-generation transplants of a mouse carcinoma, he observed islands of cartilage in sarcomatous areas of the tumor that strongly suggested to him that the sarcomatous part was of connective-tissue rather than of epithelial origin. However, since the sarcomatous areas appeared as early as the second generation of transplantation, it seems possible that the primary tumor used by Woglom may have been mixed. An apparently mixed mammary tumor of the rat developing cartilaginous areas during transplantation was described many years later by Dunning, Curtis, and Maun (*27*).

Earle (*28*) studied the Walker rat mammary carcinoma 256 *in vivo* and *in vitro*. Cultures grown *in vitro* up to 133 days, when reinoculated into rats, gave rise to tumors having an architecture typical of the parental tumor strain. However, one series, run for 175 days in the horse-serum and chick-embryo-extract culture medium, and then reinjected, produced a number of tumors simulating fibrosarcoma even though the cultures inoculated appeared to be almost entirely pure cultures of epithelial cells. The sarcomatoid structure persisted through 6 generations *in vivo*. Earle interpreted this striking change in tumor morphology as an alteration and elongation of the carcinoma cells to resemble spindle cells of sarcomas.

Ludford and Barlow (*29*) demonstrated that sarcomatous tumors originating after repeated transplantation of mammary carcinomas exhibited growth characteristics *in vitro* like those of typical sarcomas, and that all mammary carcinomas that underwent the change were powerful stimulants of fibroblastic growth *in vitro*. They considered two factors responsible for the frequency of sarcomatous transformation during transplantation of mouse carcinomas of high-cancer-strain mice: 1) a stimulation of fibroblastic growth by something liberated from the growing carcinoma cells and 2) a greater survival and persistence of stromal cells through repeated transplantations because they were isologous with cells of the new host.

Taylor and Carmichael (*30*) cultivated a mouse mammary carcinoma for 90 transplant generations, almost 3 years, in chicken eggs. Implants of the egg-grown mammary carcinoma into mice produced some tumors that resembled carcinosarcomas. In interpreting these observations, the authors postulated that normal stromal cells from the host had become malignant from contiguity with the carcinoma-cell implant and consequent infection with the mammary-tumor agent.

After examining many tumors from agent-carrying and agent-free mice and also tumors induced by the agent in low-cancer-strain mice, Dmochowski (*31*) obtained suggestive evidence of association between

the origin of a mammary tumor and its future sarcomatous transformation after repeated passages in mice. He concluded that the problem is far more complex and dependent on other factors than just the presence or absence of the mammary-tumor agent.

In more recent reports of sarcomatous change in mammary carcinomas of the mouse and rat (*32*, *33*), the authors have considered the evidence too inconclusive to establish whether "the change is due to a malignant transformation induced in the connective-tissue stroma by the carcinoma cells, whether the original tumor was mixed, or whether it represents progressive anaplasia of the carcinomatous cells so that they lose all suggestion of a glandular arrangement and assume a spindle form" (*33*).

Toolan (*34*) described sarcomatoid growths resulting from mammary-carcinoma cells that had sojourned in immune mice. Cells of a transplanted C3H mammary carcinoma were transferred back to susceptible C3H hosts after 4 to 6 days' sojourn in the subcutaneous tissues of A mice immune to them. The tumors that developed in no instance resembled the original mammary carcinoma. Instead, sarcomatoid growths composed of spindle-shaped cells with elongated nuclei, whorled in parallel bundles amidst profuse and interlacing bands of collagen, appeared. These grew more slowly than the original tumor and after several transfers took on the appearance of an atypical anaplastic carcinoma with more rounded cells and moderate amounts of reticulin and collagen. The author interpreted these spindle-shaped cells as variants of the carcinoma cells and stated that further experiments are necessary to establish whether the change is induced specifically by the immune state of the temporary host or can be duplicated in the absence of immunity.

Van Rijssel and associates (*35*) reported a genetic influence on the frequency of sarcomatoid transformation in mammary carcinomas of mice. In strain DBA the transformation was rare, whereas in strain O2O about 75 percent of transplanted carcinomas underwent the change after one or a few passages. In the latter strain, many of the spontaneous primary tumors contained sarcomatous areas. Tumors in O2O mice induced by the mammary-tumor agent grew more rapidly and showed no sarcomatous transformation, while, in the absence of the agent, the tumors grew more slowly and sarcomatous transformation was the rule.

These authors concluded 1) that slow growth of the carcinoma may be a prerequisite for sarcomatous transformation, and 2) that the transformation occurs in the carcinoma cells rather than in the connective-tissue stroma. If the environmental factors are unfavorable for growth of carcinoma cells, a gradual adaptation of the cells to the unfavorable milieu takes place, and finally, thereby, a large part of the growth energy is recovered, but not the faculty of differentiation. The second conclusion was based on two sets of observations. First, in examining the tumors for collagen and reticulin fibers, they observed few fibers and little ground substance. Second, in the growth of one of the sarcomas in tissue culture, the cells formed small membranes and projections of united cells containing round to oval nuclei more characteristic of epithelial cells

than sarcoma cells of connective-tissue origin. However, from our experience in culturing many sarcomas of connective-tissue origin, the pictures shown might characterize either carcinoma or sarcoma in culture, since the latter sometimes grow in more sheetlike form. When van Rijssel reinoculated the cultures into mice, the resultant tumors assumed the sarcomatous appearance of the cells before culture.

A transplantable mammary carcinoma of the mouse described by Ringsted (*36*) appeared to undergo a sarcomatous change in the 36th passage and by the 40th to 42d passage resembled a pure spindle-cell sarcoma. During this morphologic alteration, the growth rate of the tumor increased and remained high thereafter. However, during subsequent passages, transitions from a purely sarcomatous tissue to still larger areas of closely packed, large, polygonal cells, histologically characteristic of undifferentiated solid carcinoma, were observed. From the 60th generation on, the tumor lost all resemblance to a sarcoma and appeared typical of an undifferentiated carcinoma. When the tumor was cultured *in vitro*, the growth on the glass consisted of large, flattened, epithelial-like cells, whereas in the superficial layers the cells formed cords of more elongated fibroblast-like cells. Ringsted interprets these observations as indicating that the tumor was a carcinoma throughout its history in spite of the sarcomatous appearance during some of the generations of transplantation. Further, he believed that not only the tumor described but probably most other reported "carcinosarcomas" and "sarcomatous overgrowths of carcinomas" of the mammary gland of the mouse and rat were misinterpretations of carcinoma with spindle-shaped epithelial cells.

From a spontaneous mammary carcinoma of the mouse, Gropp and Lettré (*37*) established a strain of cells in culture which showed a purely epithelial morphology. After 18 months to 2 years *in vitro*, cultures were reinoculated into mice of the inbred strain from which the tumor originated. The resulting tumors lacked the glandular acinus structure of the original tumor and appeared like polymorphocellular carcinomas. During the 3d to 5th passages *in vivo*, the tumors tended to resemble spindle-cell sarcomas, but by the 9th passage the epithelial and glandular structure of the tumor again appeared. A transplant of the 4th passage was cultured and the cells grew slowly as elongated fibroblast-like cells. After 6 to 8 months *in vitro* and 7 transplant generations, the cultures gradually assumed a more epithelial type of growth until by the 12th month the cells again showed the epithelial form of the original cell strain. Because transitional cell forms were observed both *in vivo* and *in vitro*, Gropp and Lettré interpreted these observations as morphologic alterations of the carcinoma cells. The implantation on one hand and explantation on the other and the associated change of environment were considered the causes for the retarded growth rate and spindle-cell variation in shape of the carcinoma cells.

Against this background of experimental facts and theories, let us consider the results of the present study. The tumors resulting from the

reimplantation of tissue-culture cells into the mouse presented a variety of morphologic structures, all of which have been observed previously in histologic examinations of mammary tumors undergoing alteration during transplantation. A few tumors, when the sojourn of the cells in tissue culture had been brief, still retained the glandular structure of the original tumor. Others were composed of fusiform cells, arranged in interlacing bundles, and were morphologically indistinguishable from fibrosarcomas. In others, the cells were epithelial-like and resembled the cells of anaplastic carcinomas. Of most interest were the "mixed" tumors in which two or more of these morphologic types were blended. These suggest that the loss of differentiation as evidenced by gland formation was gradual and progressive in the tissue-culture environment and that a sudden conversion from one tumor type to another did not occur.

In the serum-supplemented chemically defined medium, the epithelial glandular architecture of the C3HBA mammary carcinoma was maintained for more than a year in culture, whereas in other cell strains from less well-stabilized tumors and cultured in the horse-serum, embryo-extract medium, continued maintenance of differentiation was never observed, and the cells either became overgrown with fibroblasts of the stroma or assumed a spindle form when returned to the mouse. Apparently the tumor cells that had stabilized with an epithelial glandular architecture during the years of transplantation *in vivo* could maintain this differentiated architecture and the high arginase activity during rapid proliferation in a suitable tissue-culture environment *in vitro*.

The chromosomes of strain 2777 cells, as well as those of the epithelial strain 2940, were studied by Levan and Hsu (*38*). Both strains were heteroploid; strain 2777 was hypotetraploid and 2940 hypertetraploid. A remarkable chromosome consisting of a long, heterochromatic central part where the centromere was located and two euchromatic end parts appeared in a number of strain 2777 cells. Apart from the big biarmed structure in 2777, all the chromosomes were of the telocentric type characteristic of mouse chromosomes. Possibly the chromosomal variations and rearrangements may account for some of the difficulty encountered in transplanting these cells from culture to the mouse as has been suggested for other cell strains *in vitro* (*39*). However, after one mouse passage, all strains were readily transplantable (*40*).

The present study has shown that in the culture of mammary carcinomas of the C3H mouse, cells of the connective-tissue stroma may undergo a malignant change and give rise to sarcomas when the cultures are reinoculated into mice, as was postulated earlier for cultured tissues of hepatoma, melanoma, and thyroid tumors (*41*). In certain cell strains the stromal cells appeared to overgrow the carcinoma cells in culture, while in other strains the two cell types persisted together during prolonged culture. The malignant character of the stromal cells was first evident after at least 8 months of culture *in vitro*, a period not widely different from that observed for such conversions in normal subcutaneous connective-tissue cells, agent-containing or agent-free, grown *in vitro* (*9*, *42*, *43*). All cultures that gave rise to these sarcomas clearly consisted of fibroblast-like cells.

Cultures of cell strain 1348 resembled the cells described by Earle (*28*). These formed almost pure epithelial cell sheets *in vitro* when cultured in the horse-serum and chick-embryo-extract culture fluid, but produced spindle-cell, sarcoma-like tumors when implanted into mice after the first 5 months of culture. The three clones derived from this strain and cultured in serum-supplemented chemically defined medium produced cystic tumors, in the margins of which were cells resembling anaplastic carcinoma. However, in one single-cell clone when grown *in vitro* and *in vivo*, both spindle-cell, fibroblast-like cells, and more rounded carcinoma-like cells were observed. On the basis of these observations, it seems probable that the cells of strain 1348 were undifferentiated carcinoma cells that had lost their capacity to synthesize arginase and that assumed different forms and produced different types of tumors when grown in the two culture media.

Two other groups of fibrosarcoma-like tumors, those produced by cell strains 2577 and 2777, may also have resulted from morphologic alterations in carcinoma cells, as mentioned earlier.

All these observations indicate that under certain conditions recognizable differentiation can be maintained in rapidly proliferating long-term cultures and that the "sarcomatous" changes in the culture cell strains resulted from two entirely different phenomena: 1) the neoplastic transformation of stromal cells in culture and 2) the morphologic alteration of carcinoma cells to resemble fusiform sarcoma cells.

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PLATE 155

FIGURE 1.—Representative area of culture of strain 1348 showing tongues of epithelial cells characterizing outgrowth during 1st few weeks after explantation of mammary tumor tissue. Culture was photographed after 5 days of culture in stock medium. Explant was embedded in chicken-plasma clot overlaid with sheet of perforated cellophane. Edge of perforation in cellophane sheet may be seen at *lower left*. × 200

FIGURES 2 and 3.—Cells of strain 1348 showing disruption of cell sheets in lysed areas of plasma clot. Observe irregular shape of cells. Margin of cell sheet may be seen at *lower left* of figure 2. Cultures of figures 2 and 3 were photographed after 9 and 23 days of culture, respectively. × 200

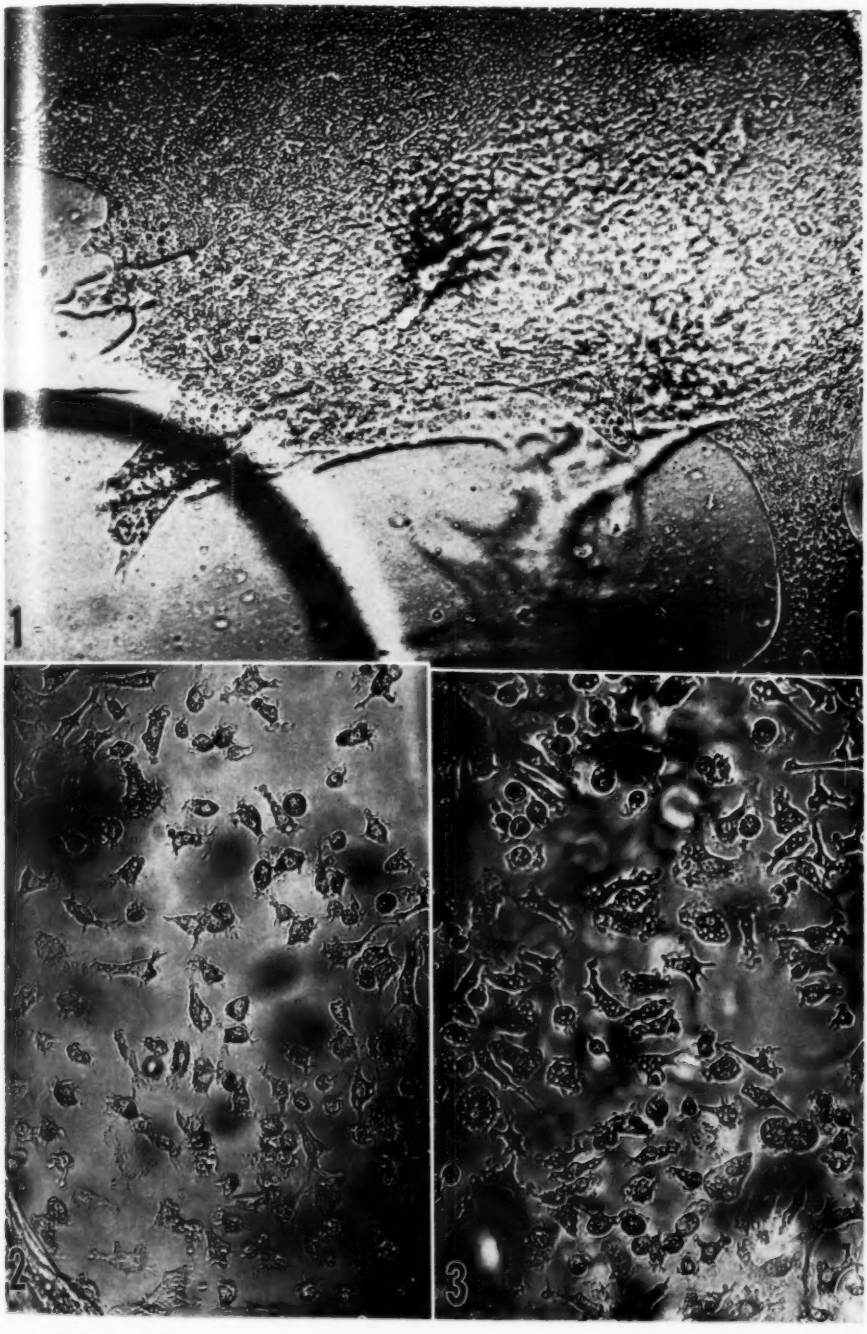

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PLATE 156

FIGURE 4.—Epithelial outgrowth from explant of C3HBA mammary carcinoma after 2 days of culture. Cells are growing in chicken-plasma clot and stock medium. × 200

FIGURE 5.—Fibroblast-like cells of strain 1350 migrating from explant of primary mammary carcinoma after 5 days of culture in same type medium as above. × 200

PLATE 157

FIGURE 6.—Culture of strain 1348 after 23 transplant generations and 21 months in culture. Cells are growing in plasma clot and form flattened epithelial-cell sheets. × 200

FIGURE 7.—Fringe of cells growing from explant of sarcoma-like growth developed from injection of 1348 cells. This culture was photographed after 5 days *in vitro* in same type medium at glass interface as above. *Note* that in comparison with figure 6, cells appear more fibroblast-like. × 200

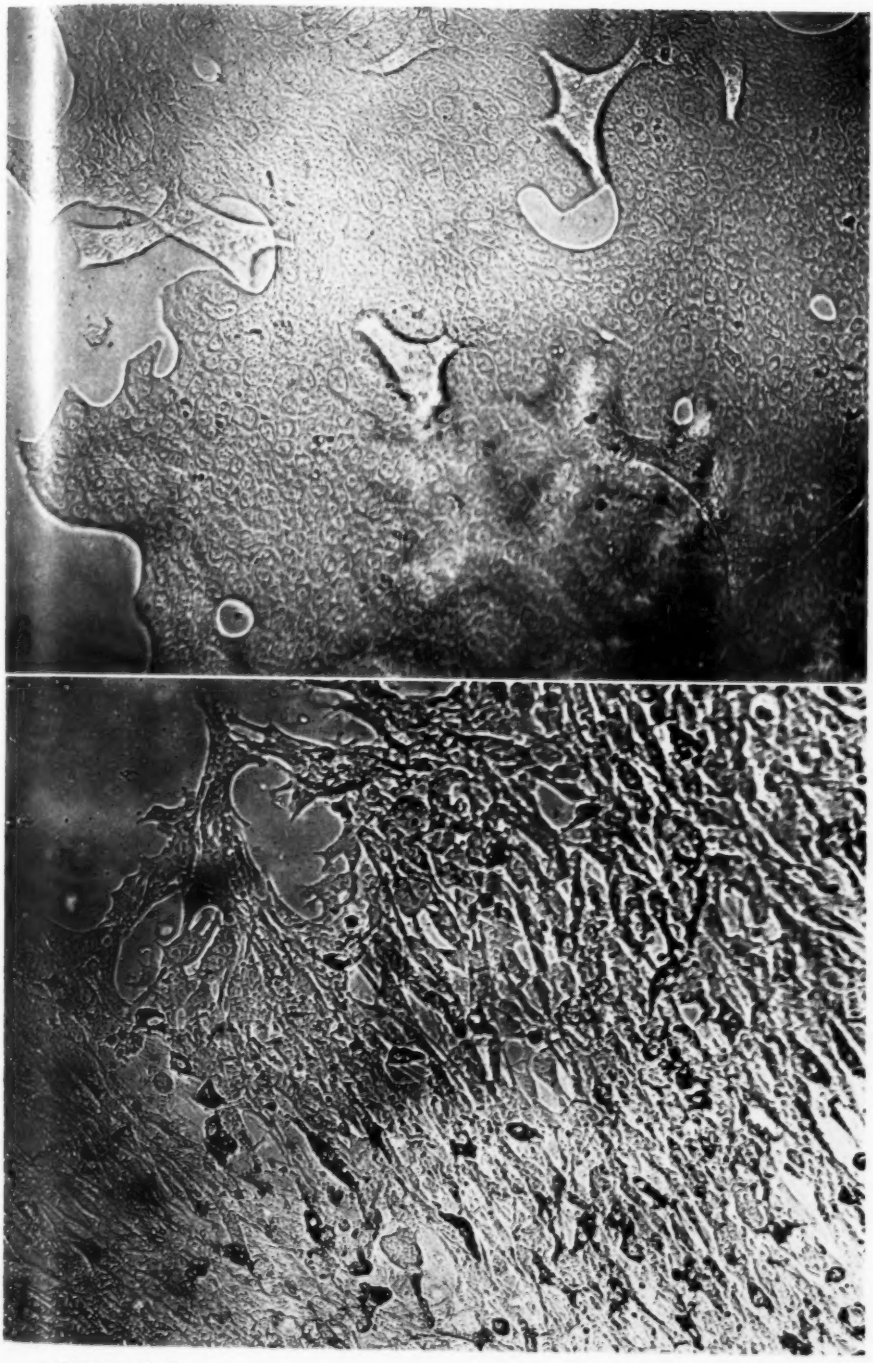

PLATE 158

FIGURE 8.—Epithelial-cell sheets at margin of culture of strain 2291 growing in plasma clot and stock medium. Culture was photographed in first generation after 34 days *in vitro*. × 200

FIGURE 9.—Strain 2291 cells after 27 transplant generations and 18 months of culture *in vitro*. Cells were now growing on glass floor of culture flask in experimental medium. × 200

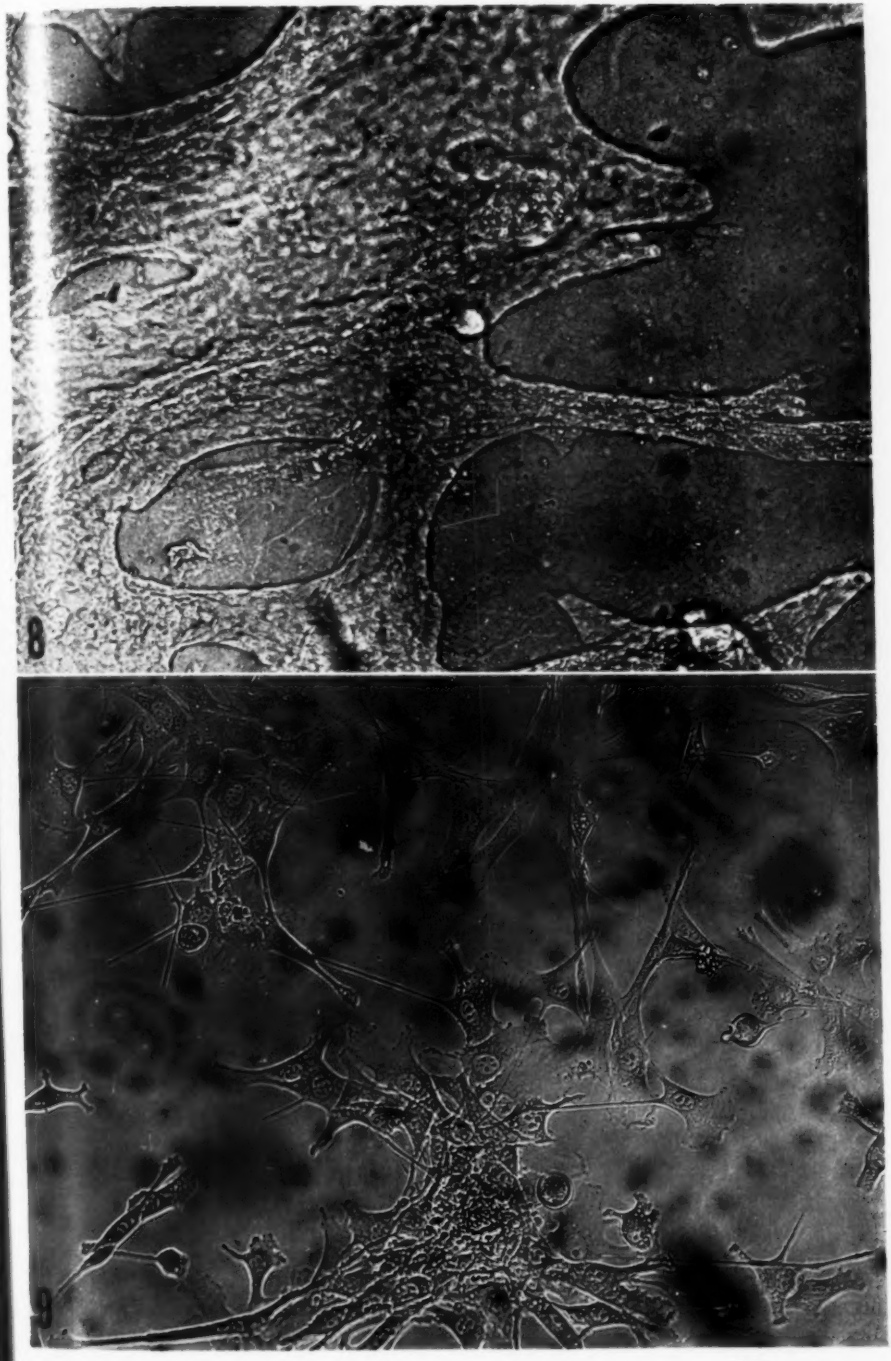
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PLATE 159

FIGURE 10.—Cells of clone 2444 emerging from open end of capillary 14 days after isolation of single cell. Cell was isolated from culture of strain 1348 after 5 years of continuous proliferation *in vitro*. There appears to be some lysis of plasma clot at capillary end. × 200

FIGURE 11.—Same clone culture as that in figure 10 photographed 7 days later. Tip of capillary may be observed in *upper left* corner. *Note* variations in cell size and shape and a few binucleate or polynucleate cells. × 200

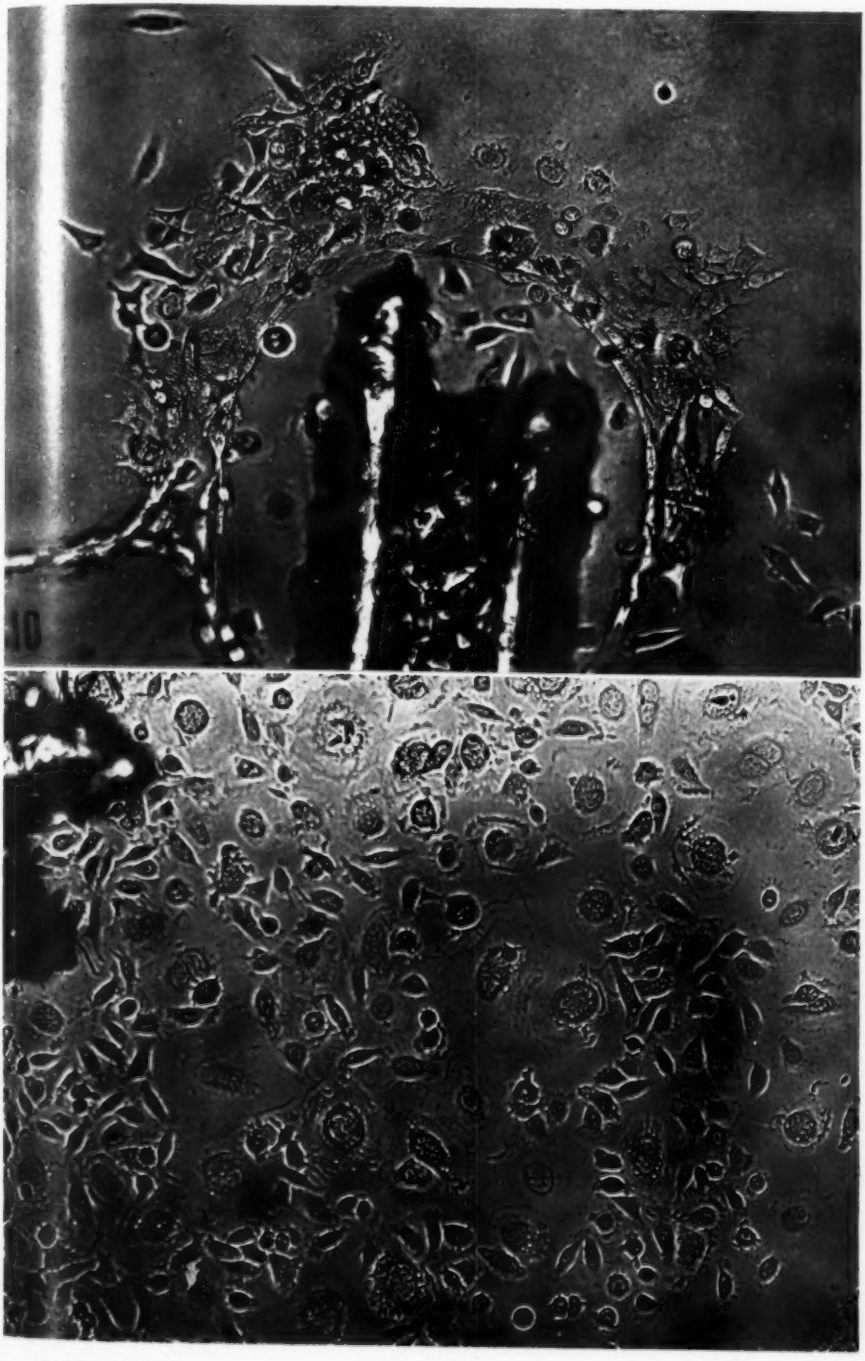


PLATE 160

FIGURES 12 and 13.—Representative areas of clone 2443 to show fibroblast-like morphology (*above*) and epithelial-like morphology (*below*). Both cultures were originated *in vitro* from same cell. Culture of figure 12 was photographed in eighth-transplant generation 4 months after single-cell isolation. Cells had been returned to stock medium and plasma clot 14 days before photographed. In this photomicrograph, *note* striking difference in morphology of cells at glass interface (*upper left*) and in clot at fluid interface (*below*). These different morphologies at different levels within same culture have been previously described for cultures of fibroblasts (*44*, *45*). However, in addition to these different morphologies within plasma-clot cultures, both fibroblast-like and epithelial-like cells were also observed in glass-substrate cultures without plasma clot. Culture of figure 13 was photographed in 33d-transplant generation a year after single-cell isolation. Cells were growing on glass floor of flask in experimental medium without plasma. × 200

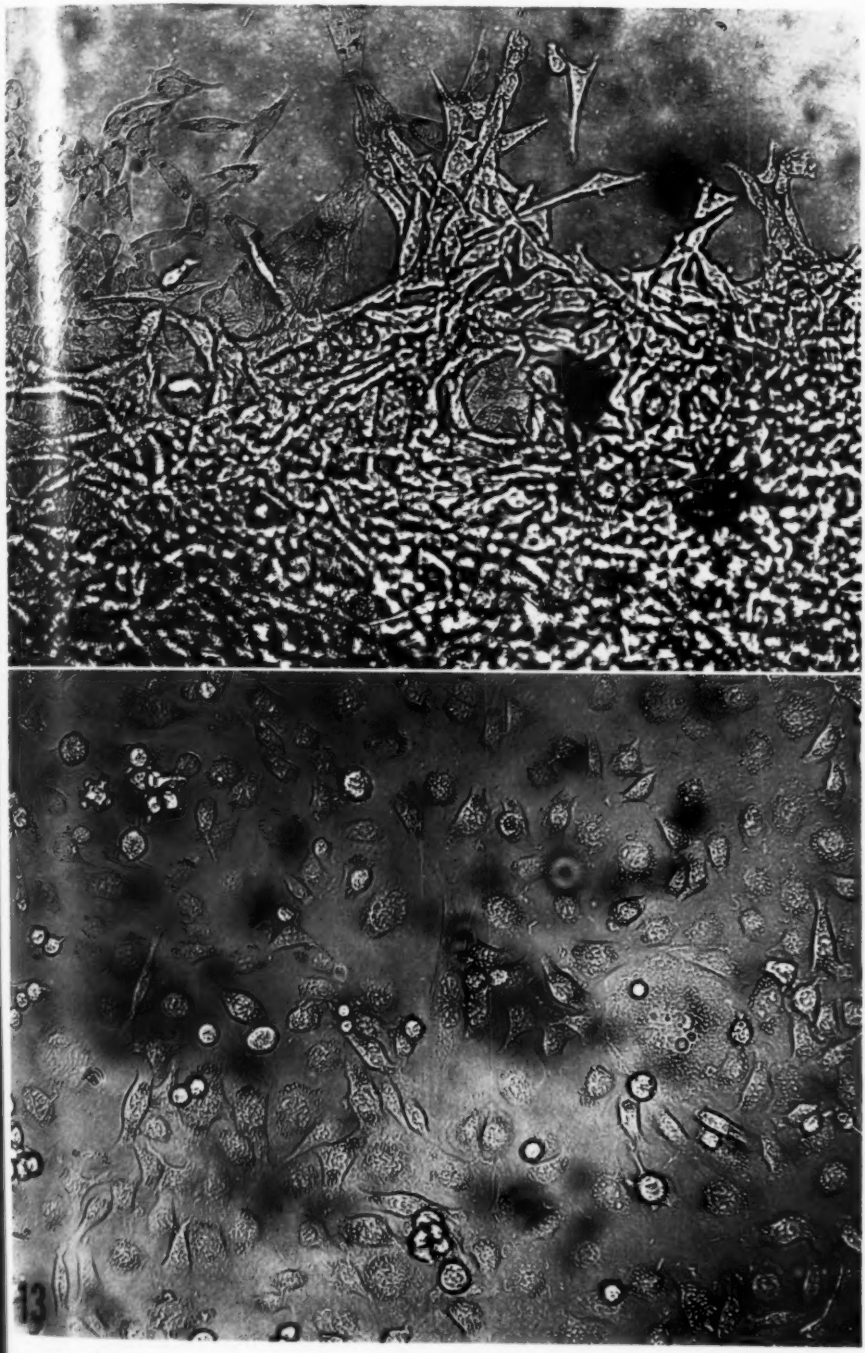
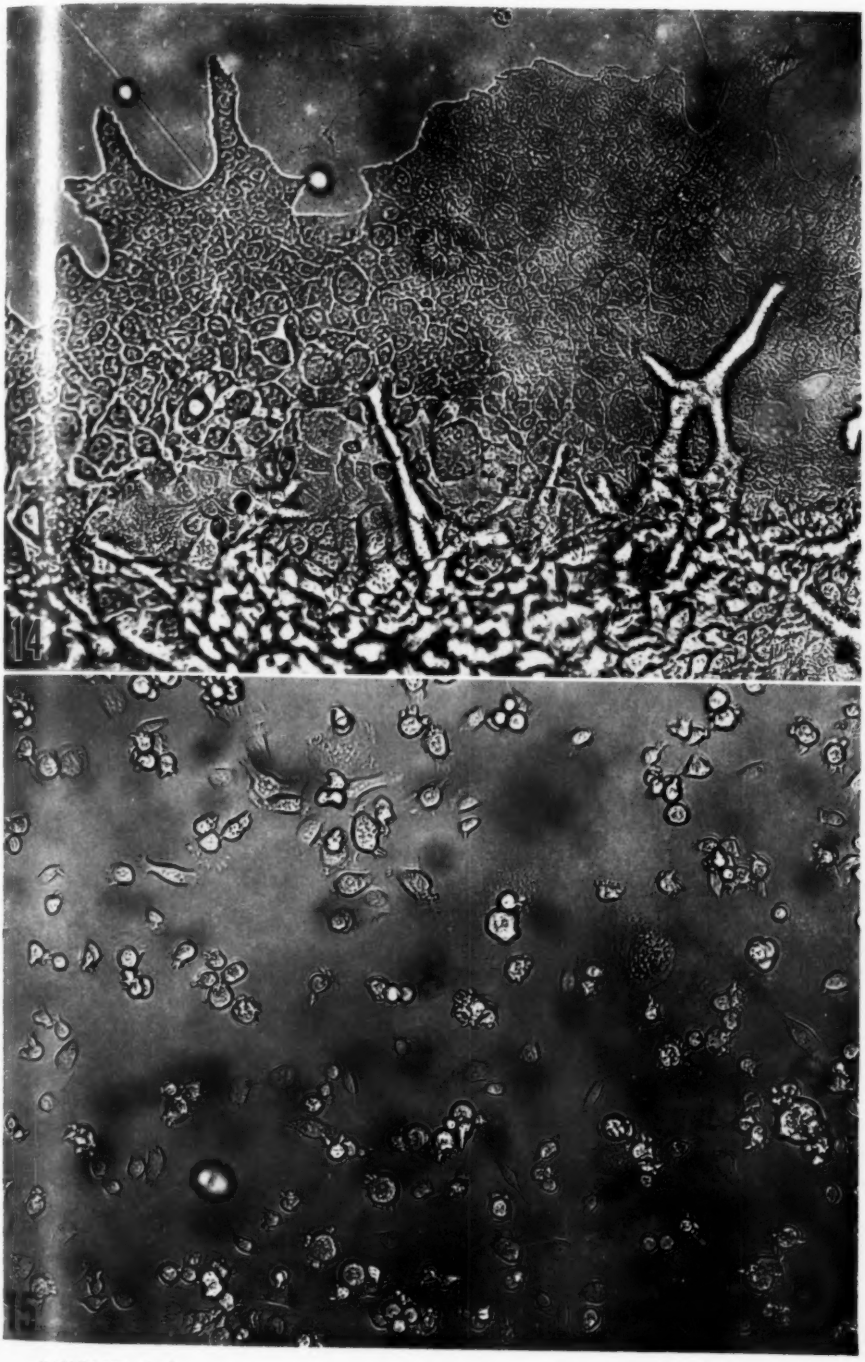


PLATE 161

FIGURES 14 and 15.—Representative areas of clone 2442 to show morphology of plasma-clot culture (*above*) and glass-substrate culture without plasma (*below*). Cells were grown in stock and experimental media, respectively. Upper continuous sheet of cells is probably at glass interface. Culture of figure 14 was photographed in fifth-transplant generation 4 months after single-cell isolation. Culture of figure 15 was photographed in the 32d-transplant generation a year after single-cell isolation. × 200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15

PLATE 162

FIGURES 16 and 17.—Cells of strain 2848 (fig. 16) and derived clone 2978 (fig. 17). *Note* morphologic similarity between clone and parental strain. Cells are growing on glass floor of flask in experimental medium. Culture of strain 2848 was photographed in 13th-transplant generation after 1 year *in vitro*. Culture of clone 2978 was photographed in 13th-transplant generation 9 months after single-cell isolation. × 200

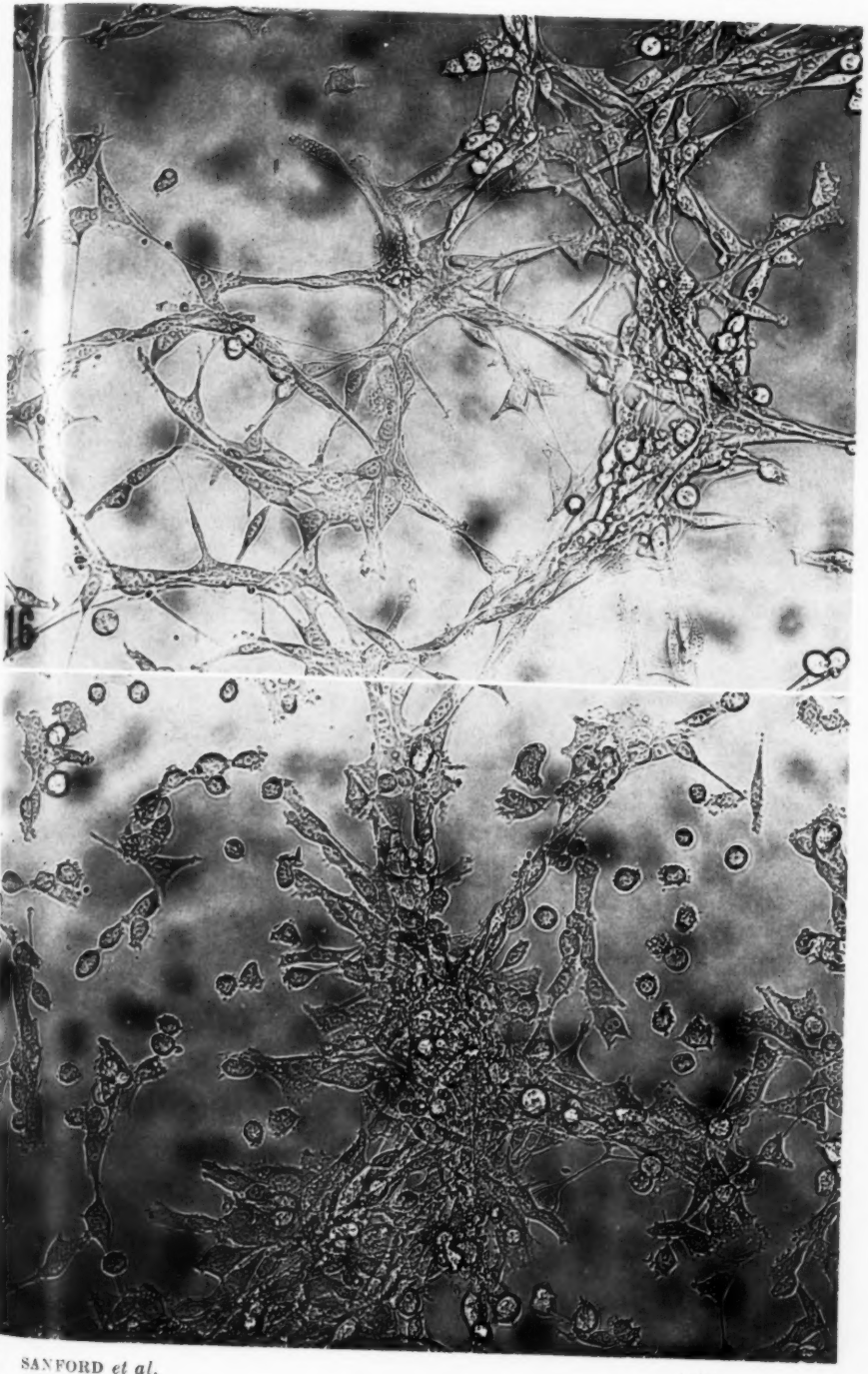
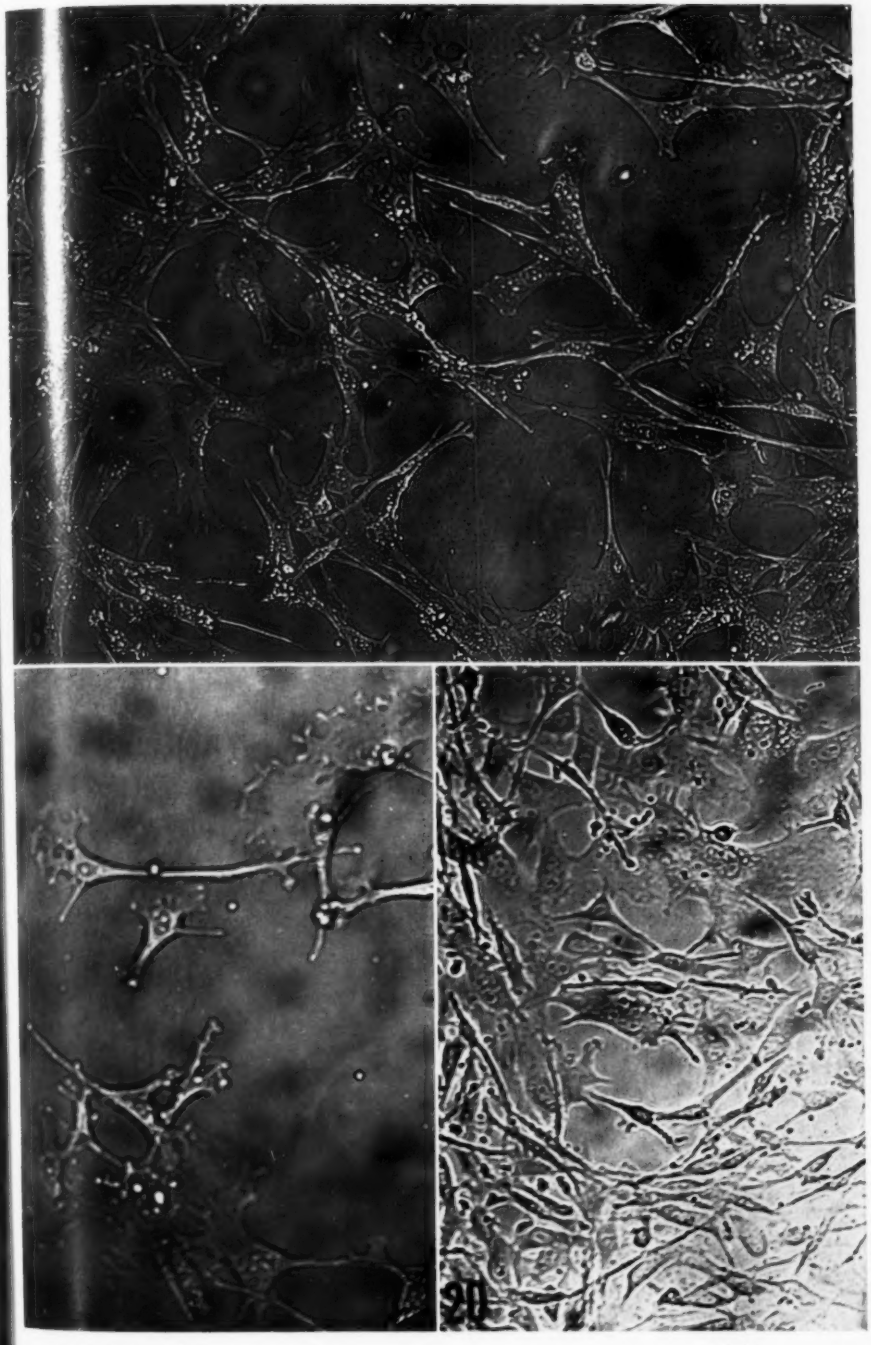


PLATE 163

FIGURE 18.—Cells of strain 2777 growing on glass floor of flask in experimental medium. Cells were photographed in fourth-transplant generation after 4 months of culture. *Note* irregular cell shape. Cells of this strain generally grew as differentiated adenocarcinomas (type B) when implanted into mice. × 200

FIGURE 19.—Cells arising from two cells of strain 2777. *Note* undulating membranes of these derivatives. These cells were photographed after 15 months *in vitro* and 9 months (9 transplant generations) after isolation of the two cells. × 200

FIGURE 20.—Cells of subline 2976 arising from 3 cells of strain 2777. These cells were photographed after year *in vitro* and 6 months (3 transplant generations) after isolation of cells. *Note* elongated, fibroblast-like cells. This cell strain when implanted into mice gave rise to sarcoma-like tumors. × 200



586399—61——13

PLATE 164

FIGURES 21 and 22.—Cells of strain 2940, growing on glass floor of flask in experimental medium. Cells were photographed in 29th-transplant generation after 22 months of culture. *Note* flattened epithelial-cell sheets. Cell sheet is so thin cells have almost same refractive index as medium and are thus difficult to see. In figure 21 observe variation in cell size and large binucleate cell (*lower center*). × 200

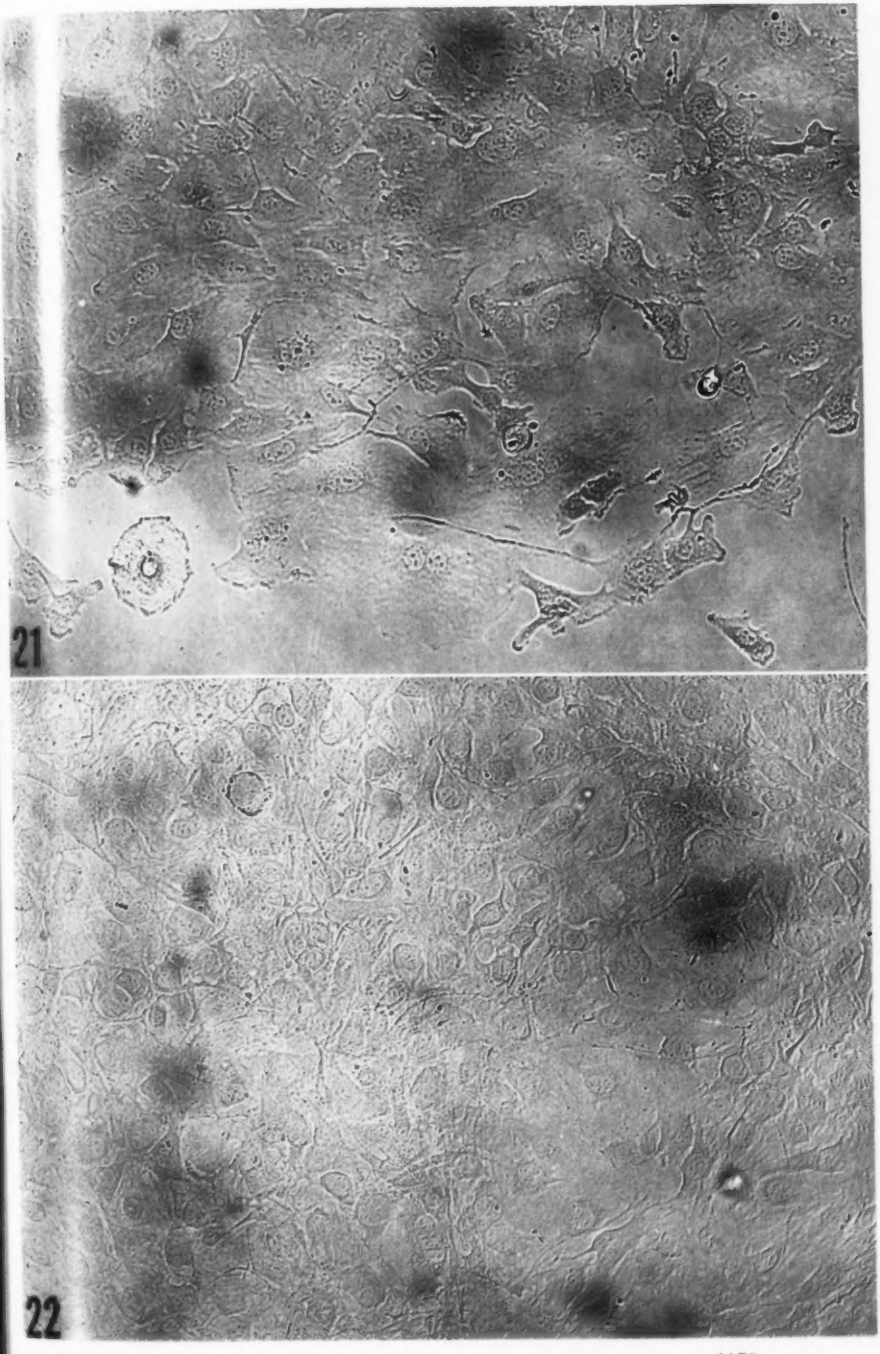

21
22

PLATE 165

All sections were stained with hematoxylin and eosin. × 290

FIGURE 23.—Mammary carcinoma, type A of basic acinar structure, resulting from injection of tissue culture derived from type A tumor grown for 3 weeks (2 transplant generations) in horse-serum, embryo-extract medium. Tumor developed in mouse 17 weeks after injection. *Note* glandular epithelial structure and resemblance to primary type A mammary tumors.

FIGURE 24.—Tumor composed of fusiform cells arranged in bundles. Tumor resembles fibrosarcoma. Cells from type A tumor were grown 40 weeks in horse-serum, embryo-extract medium. Tumor developed 10 weeks after injection.

FIGURE 25.—Tumor of glandular epithelial structure resembling adenocarcinoma type B tumor from which it was derived. Cells of C3HBA mammary tumor were grown in tissue culture for 65 weeks and transferred back to mouse. Tumor section is from second-transplant generation in mouse.

FIGURE 26.—Tumor containing clump of epithelial cells in *lower left* and glandular structure in *center* surrounded by oval anaplastic cells with more fusiform cells in *upper right*. Cells of C3HBA tumor were grown in tissue culture for 50 weeks and transferred back to mouse. Tumor section is from second-transplant generation in mouse.

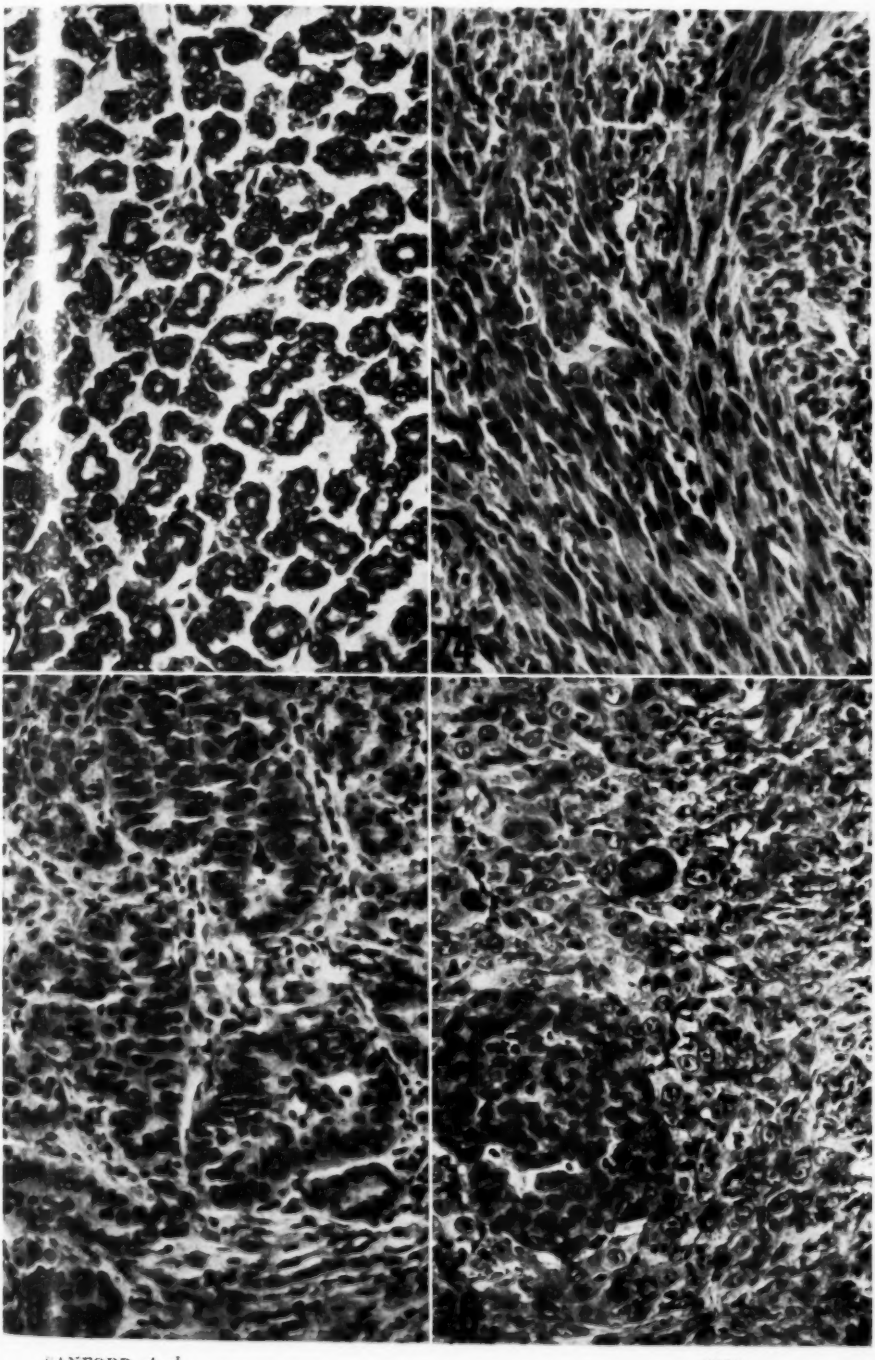


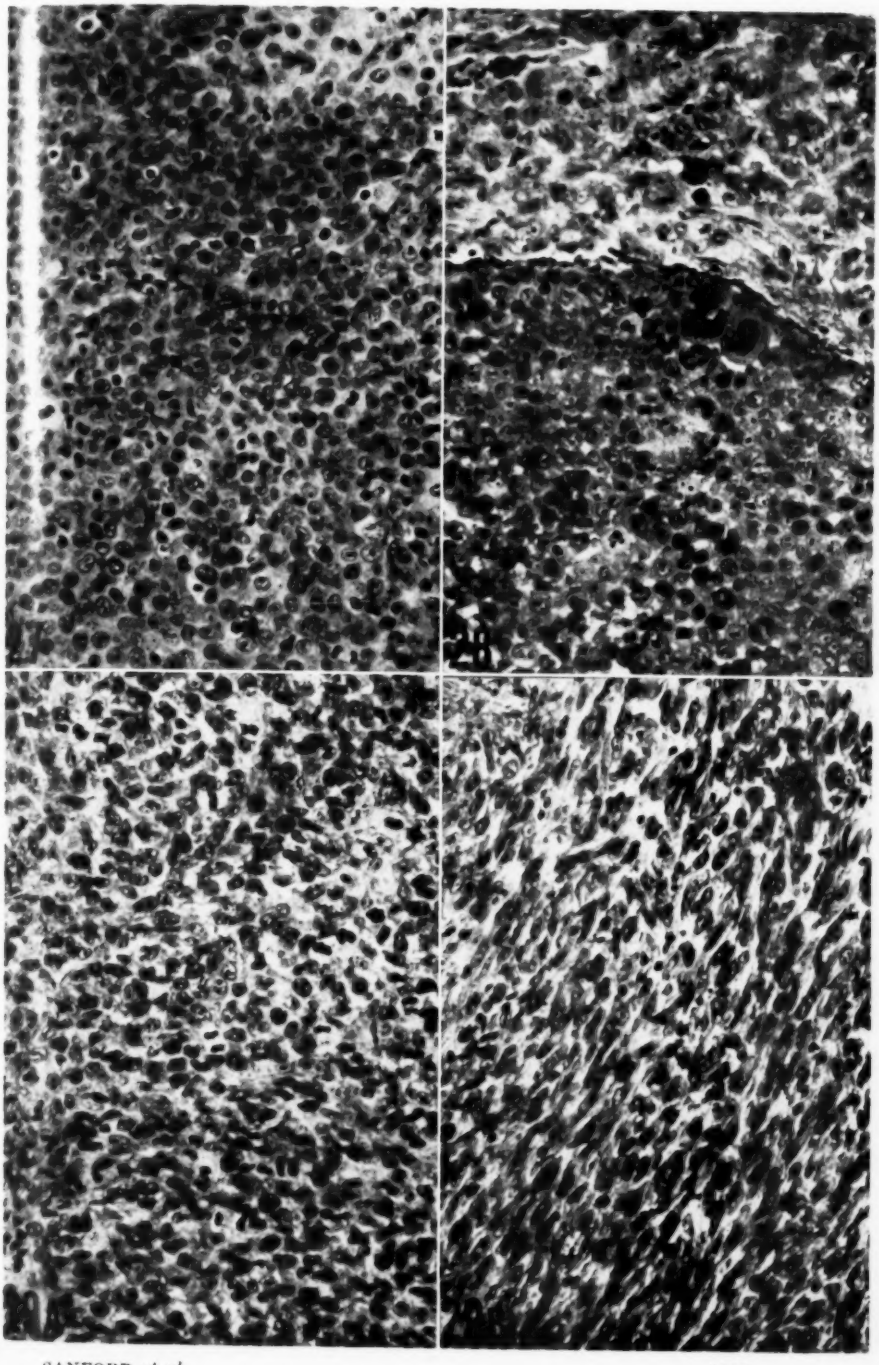
PLATE 166

All sections were stained with hematoxylin and eosin. × 290

FIGURE 27.—Tumor composed of round or oval epithelial-like cells, diagnosed as anaplastic carcinoma resembling such altered mammary carcinomas as S37 and S180. Cells from primary spontaneous type A tumor were grown in tissue culture for 303 weeks, and cells of a single-cell clone were transferred back to mouse. After 2 weeks cystic growth developed with neoplastic tissue forming the wall. Tumor section is from a second-transplant generation in mouse.

FIGURE 28.—Tumor like preceding composed of round or oval epithelial-like cells. Cells in *lower half* are more basophilic than cells in *upper half*, and tumor giant cell is occasionally found. Cells from same tumor as above were grown in tissue culture for 297 weeks, and cells of single-cell clone were transferred back to mouse. Tumor section is from third-transplant generation in mouse.

FIGURE 29, A AND B.—Two different areas from same tumor. In A, cells are generally oval and epithelial-like. In B, cells are fusiform, resembling fibroblasts. Cells were derived from same tumor as above and grown for 287 weeks in tissue culture before cells of single-cell clone were transferred back to mouse.



Maintenance of the Mammary-Tumor Agent in Long-Term Cultures of Mouse Mammary Carcinoma [1]

KATHERINE K. SANFORD, HOWARD B. ANDERVONT, GWENDOLYN L. HOBBS, *and* WILTON R. EARLE, *Laboratory of Biology, National Cancer Institute,*[2] *Bethesda, Maryland*

SUMMARY

The object of this study was to determine whether the mammary-tumor agent persists in long-term cell strains cultured from C3H mouse mammary carcinomas. Cell strains and derivative single-cell clones were established in culture from 8 different tumors of mammary-gland origin. At intervals during the period of culture, cells and supernatant fluids were assayed for the mammary-tumor agent. Variations between culture cell strains with respect to maintenance and activity of the agent were noted. In certain cell strains, some activity of the agent could be demonstrated after 6 months to more than a year of rapid cell proliferation *in vitro*. In other cell strains, the agent disappeared either through cell selection or through diminution or alteration and was no longer detectable by the test system used.—J. Nat. Cancer Inst. 26: 1185–1191, 1961.

SEVERAL ATTEMPTS have been made to cultivate or maintain the mammary-tumor agent in tissue culture. Pikovski (*1*) inoculated cultures of fibroblasts from chick heart with cell-free extracts of mammary tumors or lactating mammary glands of strain RIII mice. Certain cultures were maintained for as long as 156 days through 10 transplant generations after treatment. When extracts of these cultures were assayed in mice, 47 percent of the animals developed mammary carcinomas, as compared with an incidence of 5 percent in uninoculated controls. The test mice used were backcross hybrids obtained by crossing C57BL females to RIII males and backcrossing the (C57BL $\times$ RIII)F_1 hybrids to RIII males. A few C57BL females were also used.

Dobrynin (*2*) treated cultures of fibroblasts from connective tissue of the C57BL mouse, rat, chicken, and human embryonic fibroblasts with extracts from mammary-carcinoma tissues of strains A and C3H mice. Cultures tested for the agent after periods of 40 to 118 days after treatment showed evidence of continued maintenance of the agent.

Lasfargues, Moore, and Murray (*3*) inoculated organ cultures of embryonic mouse skin and also short-term cultures of mammary epithelium

[1] Received for publication November 9, 1960.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

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of strain C57BL mice with the agent from RIII milk. After periods of 10 days and 4 weeks, respectively, the culture cells were ground and centrifuged; the supernatant was diluted 1,000-fold and introduced into a new series of cultures. At each transfer, the tumor-inducing capacity of the culture extracts was tested in young mice of strain C57BL. Activity of the agent was maintained during 4 serial passages and 35 days in the organ cultures. The increased number of tumors produced by the extracts at each passage suggested some multiplication of the agent. However, in the cultures of mammary-gland epithelium, the agent appeared to persist for 3 months, after which no activity could be detected. In a later study (*4*) these workers found that the mammary-tumor agent could not be maintained in cultures of embryonic mouse skin, but detected a high level of activity in cultures of dissociated, prelactating mammary gland containing some of the stroma, after 3 weeks *in vitro*.

In the four studies cited, the mammary-tumor agent was inoculated into cultures of agent-free homologous or heterologous cells. In the study reported here, cells were established in culture from spontaneous, primary, and transplanted mammary carcinomas of strain C3H mice known to contain the agent (*5*, *6*). Tests were carried out to determine whether the agent is maintained during rapid proliferation of the cells in culture.

PROCEDURES

Source of cells and culture procedures.—The source of cells and culture procedures have been described (*7*). Two types of fluid culture medium referred to as "stock" and "experimental" were used. "Stock medium" consisted of a filtered mixture of 40 percent horse serum, 20 percent chick-embryo extract (1:1.25), and 40 percent Earle's saline. "Experimental medium" consisted of chemically defined medium NCTC 109 supplemented with 10 percent horse serum (*8*, *9*).

Methods of assay for mammary-tumor agent.—Two different methods of assay were used. In one, the test materials were injected intraperitoneally into agent-free susceptible female mice 1 to 3 weeks of age. The test materials from tissue culture were cells suspended in supernatant culture fluids in which large numbers of cells had been growing for 48 to 72 hours. Each mouse received 0.2 to 0.5 ml of the cell suspension. Tumor tissue assayed for presence of the agent was first minced with scissors, ground with sand in a mortar, and then diluted with 3 to 5 ml Locke's or Earle's saline. After the particles had settled, 0.2 ml of the supernatant was injected intraperitoneally into each mouse. The following agent-free mice were used, all from Dr. Andervont's colony: C3Hf, (BALB/c $\times$ DBA/2f)F_1, referred to hereafter as (C $\times$ DBA/2f)F_1, (C57BL $\times$ I)F_1, and (C57BL $\times$ I)IBC, referred to hereafter as (B $\times$ I)F_1 and (B $\times$ I)IBC, respectively. When mature, each female was mated to an agent-free male of the same type, allowed to have one litter, and examined every 2 weeks thereafter for development of tumors. These mice have a very low incidence of spontaneous mammary tumors, considerably less than 5

percent, especially if they have only one litter. If a tumor developed, the mouse was killed and sections of the tumor were fixed and stained as described (*7*). Tumors were diagnosed as types A, B, or C according to the classification of Dunn (*10*). Production of type A or B mammary carcinoma was considered presumptive evidence for presence of the mammary-tumor agent in the test materials.

In the second method, the test cells were placed in a diffusion chamber which was then inserted into the peritoneal cavity of a 5- or 6-week-old female mouse of strain C (BALB/c AnN). The diffusion chambers were constructed of HA cell-impermeable Millipore filters as described by Algire and associates (*11*).[3] For implantation in some of the younger mice, we reduced the size of the chamber by sealing the filters to only the smaller of the plastic rings. Since these mice were not mated, it was necessary, in tests for the mammary-tumor agent, to provide estrogenic stimulation. At 9 to 10 weeks of age, a pellet of diethylstilbestrol (5 or 10%) in cholesterol prepared as described by Shimkin and White (*12*) was implanted subcutaneously by trocar into the right axillary region of each mouse. Mice were observed for tumors as described earlier.

RESULTS

The 8 cell strains and 5 derivative clones were examined for presence of the agent after different periods of culture. Results of these tests are summarized in table 1. The first 6 cell strains, all from primary tumors, were tested after 16 to 26 weeks (up to 11 transplant generations) of growth in stock medium. Of these 6 strains, 2 showed no activity of the agent. However, one of these (strain 1335) was assayed in only 3 mice, and the other (strain 1349) originated from tumor tissue that, likewise, showed no activity. The other 4 strains exhibited activity of the agent. Mammary tumors appeared in 17 of 36 mice receiving injections of cultures of these 4 strains; 8 of the tumors were of type A, 7 of B, and 2 of C. The results thus indicated that the mammary-tumor agent can be maintained in the rapidly proliferating tumor cells for as long as 26 weeks or up to 11 transplant generations of growth in stock medium.

One of the 6 cell strains, 1348, was continued in culture for longer than 26 weeks, and after 5 years, 3 single-cell clones were developed. The three clones (2442, 2443, and 2444) as well as the parental strain were examined for agent. Of 144 mice receiving injections of supernatant fluids, or in 2 with tumor tissue (*see* footnote, table 1) from 34 cultures, only one mouse developed a mammary tumor. This tumor arising after 17 months was diagnosed as type C, which most often appears in old female mice without the agent and is rare in mice with the agent (*10*). Therefore, this tumor could not be considered evidence for presence of the agent. It was concluded from these data that the mammary-tumor agent, present during the first 26 weeks of culture, either disappeared

[3] The authors express their appreciation to Dr. Ruth Merwin and Mr. Roy Moore of this Institute for their helpful advice and for some of the diethylstilbestrol preparations used in these tests.

TABLE 1.—Tests for mammary-tumor agent in four of the original tumors* and in the culture strains and clones

Cell strain or clone	Weeks in culture	Number of cultures assayed	Mice used for assay	Results of assay †	Individual tumor latent periods (months)	Tumor type ‡	Average age of mice dying without mammary tumor (months)
1333	19–20	3	(C × DBA/2f)F_1	4/6	5, 8, 9, 11	A(2)B(2)	24
1335	19–20	2	(C × DBA/2f)F_1	0/3	—	—	24
1348	0	—	(B × I)F_1	1/2	10	—	21
			(B × I)IBC	1/3	11	—	21
	16–26	7	(C × DBA/2f)F_1	3/14	9, 18, 23	A(2)B	21
	40–98	26	C,(C × DBA/2f)F_1	0/96	—	—	21
Clones:							
2442	29	2	C3Hf	0/11	—	—	20
2443	29–46	4§	C, C3Hf	0/24	—	—	19
2444	29	1	C	0/3	—	—	17
		1	C3Hf	1/10	17	C	21
1349	0	—	(B × I)F_1(B × I)IBC	0/6	—	—	20
	17–26	3	(C × DBA/2f)F_1	0/6	—	—	24
1351	0	—	(B × I)F_1	1/4	15	—	21
			(B × I)IBC	0/2	—	—	21
	17	2	(C × DBA/2f)F_1	2/3	21, 22	C, A	24
1352	0	—	(B × I)F_1	2/3	10, 15	—	21
	16–26	6	(B × I)IBC	2/2	16, 21	—	—
			(C × DBA/2f)F_1	8/13	8, 9(2), 12, 13, 14, 15, 23	A(3)B(4) C	24
2940	50	1¶	C	2/7	9, 23	A(2) B(2)	17
2777	50–65	5¶	C	1/11	9	B	13
Clones:							
3025	48	1¶	C	0/9	—	—	17
3026	44–48	2¶	C	0/5	—	—	14

* The original tumors are indicated as "0" weeks in culture.

† Number of mice with mammary tumors divided by number of mice used for assay.

‡ A = mammary tumor type A of basic acinar structure; B = mammary tumor type B of variable epithelial glandular pattern; C = mammary tumor type C composed of small cysts lined by cuboidal epithelium. Number of mice developing tumors of each type is indicated in parentheses.

§ Two of the 4 cultures of clone 2443 and all cultures of strain 2777 were inoculated into agent-free C3Hf mice, and the resulting tumors were tested for agent after 1 or 3 passages in C3Hf mice.

¶ Cells tested in diffusion chambers in peritoneal cavity.

entirely from the cells of this strain or was so reduced in amount or altered as to be inactive in these tests.

The last 2 cell strains, 2940 and 2777, originated from transplanted tumors carried for 2 and 186 generations, respectively, in agent-containing mice. Strain 2777 was grown from a transplant of C3HBA mammary tumor (*13*). After 25 weeks of culture, 2 single-cell clones (3025 and 3026) were established from strain 2777. Cells of the two strains and clones were assayed for the agent with the diffusion-chamber technique. Cells were tested after a year (7 to 13 transplant generations) of culture in experimental medium, and cells of strain 2777 were grown in agent-free C3Hf mice as tumors for an additional 4 months before tested. Of 7 mice implanted with diffusion chambers containing cells of strain 2940, 2 developed mammary tumors, one of type B, and the other, multiple tumors, 2 of type A and one B. Of 25 mice implanted with cells of strain 2777, 1 mouse developed a tumor of type B and a second mammary tumor of unusual morphology consisting largely of dilated ducts with inspissated hyaline material. All 3 mice had received a pellet of 5 percent diethylstilbestrol. No mammary tumors of type A or B developed in the 94 control mice,[4] each implanted with a diffusion chamber and given a pellet of 10 percent diethylstilbestrol.

Andervont, Dunn, and Canter (*14*) found strain C virgin females particularly resistant to diethylstilbestrol-induced mammary tumors. According to their report of 53 females, each implanted with a 2.5 or 5 percent diethylstilbestrol-cholesterol pellet, not 1 developed a mammary tumor, though the mice lived to a mean age of 21 months. In view of these data, it seems probable that the tumors arising in the strain C mice resulted from a small amount of mammary-tumor agent persisting in the cultured cells.

DISCUSSION

In growing mammary-tumor cells *in vitro*, we hoped that the conditions of culture outside the organism might promote rapid propagation and release of agent, which would thus allow accurate quantitative studies on factors influencing synthesis and replication of the mammary-tumor agent. However, the results of this study reveal maintenance of the agent at a low level of activity, with presumably a limited degree of multiplication during the numerous transplant generations *in vitro*. Variations between culture cell strains with respect to maintenance and activity of the agent have been noted. In certain cell strains activity of the agent was demonstrable after 6 months to more than a year of rapid cell proliferation in culture. In other cell strains, the agent disappeared either through cell selection or through diminution or alteration and was no longer detectable by the test system used. No activity was observed in cell strains that produced fibrosarcoma-like tumors when implanted in mice (7). Although the cells were cultured in media containing serum taken from geldings

[4] These mice were from unpublished experiments by Dr. Ruth Merwin of this Institute.

which would contain some estrogens, the possible effects of controlled hormonal stimulation on cell differentiation and propagation of agent were not explored.

All the long-term strains of mammary-carcinoma cells tested for agent maintained their malignant properties when reimplanted into mice (*7*). In consideration of the relationship of the mammary-tumor agent to maintenance of the malignant state of the cell, the results obtained here during serial propagation of the cells *in vitro* resemble those obtained during serial propagation of the cells in the yolk sac of chick embryos or in agent-free mice.

Armstrong and Ham (*15*) cultured mammary-carcinoma tissue from a strain C3H mouse in the yolk sac of chick embryos, harvested the tumor tissue every 13 days for further inoculations, and detected the agent in the tumor cells after 31 such passages (58 weeks) in the yolk sac. Andervont (*16*) failed to detect any agent in the transplanted cells of a spontaneous mammary carcinoma after the third and 23d passage in agent-free hosts, even though the primary tumor originating in a strain C3H female contained demonstrable agent. However, several workers did recover the agent from transplanted mammary tumors after 10 (*17–19*), 30 (*20, 21*), and 42 (*22*) serial passages in mice which did not themselves carry the agent. In one of these studies (*20*), activity of the agent had decreased in the 18th passage, and the agent could be demonstrated thereafter only from extracts of high concentration. In another (*22*), the agent was detected in tumor cells of the 42d generation but not in transplants of the 86th generation (*23*). In an additional study[5] of a mammary carcinoma originating spontaneously in a C57BL mouse, foster-nursed by a C3H female and carried in agent-free mice, the agent was detected in transplants of the 9th, 11th, and 19th generations but not in transplants of the 45th, 71st, and 100th generations, even though very susceptible strain C3Hf young mice were used for the tests.

It appears from these observations that the mammary-tumor agent may persist and presumably multiply within the tumor cells for an extended period during serial passage in the yolk sac of chick embryos or in agent-free mice. However, the tumor cells can apparently continue to grow autonomously even after the agent has either disappeared or become so altered or reduced in amount as to be no longer detectable in the cells. The present results on mammary-carcinoma cells in long-term culture also support this conclusion.

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Alterations in Morphology, Arginase, and β-Glucuronidase Within a Clone of Mouse Tumor Cells *In Vitro*[1]

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SUMMARY

These studies describe the development *in vitro* of hereditary differences in morphology, further alterations in arginase activity, and also apparently β-glucuronidase activity within a single-cell clone of mouse tumor cells. The difference in arginase activity persisted during long-term culture of the cells in different media *in vitro* as well as during growth of the cells as tumors in mice. Additional hereditary alterations in morphology and level of arginase activity within the tumor-cell clone have been identified by means of clonal analysis. Data are presented on the tumor-producing capacity of the cells, the arginase activity of the tumors grown from the tissue-culture cells, the arginase activity of clonal derivatives, the β-glucuronidase activity, the chromosomal constitution of the cells *in vitro*, and certain other biochemical properties distinguishing these cells from some other strains of cells grown for long periods under similar culture conditions *in vitro*.—J. Nat. Cancer Inst. 26: 1193–1219, 1961.

ONE OF the first reports of an enzymatic difference arising *in vitro* within a single-cell clone of mammalian tissue cells appeared from this laboratory in 1958 (*1*). In the examination of a number of mammalian cell strains in culture for activity of certain enzymes, a significant difference in arginase activity between two lines of mouse tumor cells derived in culture from one cell was observed. Cells of one line showed an activity 20 times and the other line 400 times the activity of the parental line from which the tumor clone originated. The present report supplies further evidence that this difference in arginase activity is a hereditary property of the cells and persists not only during years of proliferation in different culture media *in vitro* but also during growth of the cells as tumors in mice. In addition, certain other hereditary alterations within this clone will be described and a few biochemical properties that distinguish this clone from some other strains or clones of C3H mouse cells

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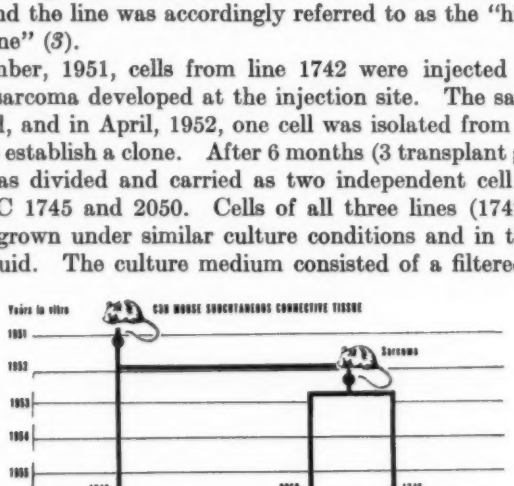
[4] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

isolated from different tissues and grown for long periods under similar culture conditions *in vitro*.

ORIGIN AND HISTORY OF CLONE

The origin and history of the tumor-cell clone are outlined in text-figure 1. In November, 1950, a strain of cells was originated in culture from an explant of subcutaneous connective tissue taken from an adult male mouse of strain C3H/He [5] (*2*). Three months later, in March, 1951, one cell was isolated from a culture of this cell strain to establish a clone which was divided and carried as a number of independent cell lines. By 8 months later, cells of one of these lines, designated NCTC 1742 (text-fig. 1), had undergone a malignant change and regularly grew as tumors when implanted into C3H mice. The tumors were diagnosed as sarcomas, and the line was accordingly referred to as the "high sarcoma-producing line" (*3*).

In November, 1951, cells from line 1742 were injected into a C3H mouse. A sarcoma developed at the injection site. The sarcoma tissue was cultured, and in April, 1952, one cell was isolated from the sarcoma cell strain to establish a clone. After 6 months (3 transplant generations), the clone was divided and carried as two independent cell lines, designated NCTC 1745 and 2050. Cells of all three lines (1742, 2050, and 1745) were grown under similar culture conditions and in the same lots of culture fluid. The culture medium consisted of a filtered mixture of



TEXT-FIGURE 1.—Origin and derivatives of mouse-tumor clone. Genealogical details and additional cell lines (*2, 4*) have been omitted to simplify the presentation. "Stock medium" refers to a mixture of horse serum, chick-embryo extract, and balanced saline. "Experimental medium" is the chemically defined medium NCTC 109 supplemented with 10 percent horse serum (*5, 6*).

[5] This cell strain is not related to strain L, which originated 10 years earlier from a different C3H mouse.

horse serum, chick-embryo extract (1:1.25), and Earle's saline prepared as described previously (*7*).

In April, 1958, certain cultures from lines 2050 and 1745 were transferred to chemically defined medium supplemented with 10 percent horse serum (*5*, *6*). The two new lines were designated NCTC 3069 and 3074, respectively, and the original two lines were closed.

Previously, a single-cell clone NCTC 2472 from the high sarcoma-producing line had been established in the serum-supplemented chemically defined medium (*8*). Two other clones, NCTC 3423 and 3610, were isolated in April and December, 1959, from line 3074. Clone 3610 is referred to as a clone, although when first observed 17 hours after isolation, 2 elongated cells were present. These appeared to be attached as though the division of one cell had just occurred.

PROCEDURES

Culture procedures.—Cells were grown under perforated cellophane (*9*) in Carrel D-3.5 flasks containing 1 ml of culture fluid. The fluid medium was renewed thrice weekly at which times the culture was gassed with a humidified mixture of 5 percent CO_2 in air to adjust the *p*H, and the flask was sealed with a silicone stopper. Cells were subcultured when the cell sheet covered the floor of the flask, usually at 1- to 2-week intervals. In subculture, the cells were shaken from the substrate, and the cell suspension transferred by pipette to 2 or 3 flasks. Neither trypsin nor antibiotics were used. To harvest more cells, we transferred them to T-60 flasks (*10*), where they were grown in 8 to 15 ml of culture fluid on the glass floor of the flask.

Cloning procedure.—Clones were established from single isolated cells by procedures described (*11*, *12*). All solutions, including the chicken plasma, were filtered through a #03 porosity Selas filter before use.

Photographic records.—Photographs of randomly selected cultures were made at intervals by means of photomicrographic procedures described (*13*). Cultures of the living cells were photographed 24 hours after a renewal of culture fluid.

Procedure for injecting culture cells into mice.—Cells were aseptically injected (*14*) as a suspension in 1 ml of culture fluid into the thigh muscles of 1- to 4-month-old male mice of strain C3H/HeN. Some of these mice, as indicated later, were X-irradiated with a whole-body dose of 425 r before injection.[6] Physical factors were 200 kv, 15 ma, 120 r per minute at a focal distance of 54 cm through 0.25 mm Cu and 0.55 mm Al filters. The inoculum size was estimated by the enumeration of cell nuclei (*15*) in two 0.2 ml aliquots of each cell suspension. To measure the tumor latent period, we examined mice weekly for any palpable growth at the injection site. The tumor latent period measured the interval from injection of cells to the appearance of a palpable tumor at the injection

[6] The authors express their appreciation to Mr. Henry Meyer, of the Radiation Branch of this Institute, who irradiated the mice.

site that continued progressive growth. Diagnosis of sarcoma was made by microscopic examination of tumor sections fixed in Zenker-formol and stained with hematoxylin and eosin and with van Gieson's picrofuchsin. Mice that failed to develop tumors were kept under observation for 3 to 4 months before autopsy.

Procedure for chromosome studies.—The procedures for chromosome studies were similar to those described earlier (*16*).

Chemical methods.—Arginase activity was estimated as previously described with activation of the enzyme by 0.05 Mn^{++} for 4 hours prior to the assay (*1*).

Glycogen was estimated in cells from culture and in the tumors by use of the anthrone reagent (*17*). The tissue was digested with 5 N KOH for 1 hour, and ethyl alcohol added to a final volume of 56 percent. After centrifugation, the precipitate was treated with cold 5 percent trichloroacetic acid. After another centrifugation, the supernatant extract was decanted, and the solution treated with 5 volumes of ethyl alcohol. After standing for an hour, the precipitate was thrown down in the centrifuge, the liquid decanted, and the tubes allowed to drain. The precipitate was taken up in water and the tube again centrifuged. The aqueous solution was decanted, and the anthrone reagent applied (*18*).

For the determination of β-glucuronidase activity, cultured cells were cooled to 0° C in the presence of culture medium, sedimented by low-speed centrifugation, washed briefly in cold 0.25 M sucrose, and finally homogenized in a volume of this medium equal to 19 times the volume of packed cells. In order that the full activity of the enzyme might be subsequently observed, aliquots of the homogenates were preincubated at 37° C for 2 hours in 3 volumes of 0.01 M acetate buffer, *p*H 5.0 (*19*). The activity of the preincubated material was then determined by a modification of the method of Talalay, Fishman, and Huggins (*20*). The reaction mixture (vol. 1.5 ml) contained 0.0005 M phenolphthalein glucuronide (Sigma Chemical Co., St. Louis, Mo.) and 0.04 M acetate buffer, *p*H 5.0. The reaction was initiated by the addition of enzyme and stopped after 60 minutes at 37° C by the addition of 2.5 ml of 0.4 M glycine buffer, *p*H 10.4. The optical densities of the centrifuged mixtures were read at 550 mμ, and converted to μg of free phenolphthalein after corrections for the appropriate tissue and substrate blanks. Reaction rates were found to be linearly proportional to time and enzyme concentration under the conditions of assay.

Total nitrogen was determined by nesslerization of acid digests of the homogenates.

EXPERIMENTS AND RESULTS

Morphologic Changes in the Cells

During the 1st year after single-cell isolation, cells of both lines were indistinguishable in morphology from the elongated, fusiform cells (*2*) of

the high sarcoma-producing line (figs. 1 and 4). Also, tumors developed from these cells in strain C3H mice resembled in morphologic pattern the sarcoma from which the clone originated (figs. 7, 8, and 9).[7] These observations seemed to identify the clone as descended from a cell of the high sarcoma-producing line. Notable amounts of collagen were found in some of the tumors produced; in others there was less collagen and the cells appeared rounded. However, differences between tumors produced by cells of each line were so slight as to be considered negligible.

By 16 months after single-cell isolation, in August, 1953, cells of line 1745 appeared less elongated and more irregular in size and shape (fig. 2). By 48 months after single-cell isolation, in April, 1956, rounded granular cells predominated in the culture, though occasional elongated forms and large cells were observed. This altered morphology stabilized and has persisted for the past 4 years (fig. 3).

Because the cell population of line 1745 seemed to stabilize with at least two different cell types present, a clonal analysis was carried out to determine whether these different forms were modulations of one basic cell type or were genetically different. The two clones 3423 and 3610, as well as another cell line originating from 3 cells (figs. 14, 15, 16, and 17), revealed the presence of two distinctly different cell types, one elongated and fibroblast-like similar to cells of the original high sarcoma-producing line and the other more rounded and somewhat epithelial-like. These different morphologies have persisted in the derivative clones.

When the morphologic instability in line 1745 cells was first noted, some cells of line 2050 appeared less elongated, more irregular in shape, and more granular. Two years later, the altered cells seemed to have completely populated the cultures (fig. 5), and line 2050 was so altered that the cells were not recognizable as fibroblasts but appeared more like cells of epithelial origin. This morphologic pattern has persisted (fig. 6).

The tumors produced by line 2050 cells injected into mice in November, 1954, and October, 1955 (31 to 42 months after single-cell isolation), differed from those produced earlier and from those growing from line 1745 cells (figs. 9 and 10). The cells were rounder. There was less collagen formation, and the growths were hemorrhagic and less firm. At first this alteration appeared in only a few of the tumors. Later, cells of line 2050 regularly produced this type of tumor (fig. 11). By February, 1958, tumors produced by cells of line 1745 also showed the same type of alteration (fig. 12).

Metastases were not observed except to the liver and lungs of two mice, irradiated and receiving injections of line 1745 and line 3069 cells, respectively (figs. 12 and 13).

The growth of cells in serum-supplemented chemically defined medium (derivative lines 3069 and 3074) appeared not to influence either their morphology *in vitro* or the morphology of tumors produced by the cells injected into mice.

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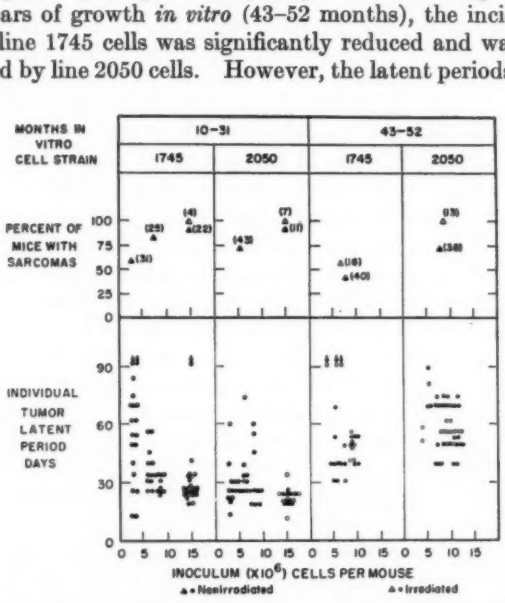
[7] The authors are indebted to Dr. Thelma B. Dunn, of the Laboratory of Pathology of this Institute, who examined the tumor sections and described and classified them as to morphologic pattern.

In summary, cells of both lines resembled cells of the high sarcoma-producing line during the 1st year in culture but showed alterations in morphology after more prolonged growth *in vitro*. The alterations were sufficient to influence the morphologic pattern of the tumors produced by implantation of the cells into mice.

Tumor-Producing Capacity

The capacity of the cells to grow as sarcomas in C3H mice was determined over a 4-year period (text-fig. 2). During the first 3 years in culture (10–31 months), there was little difference between the two lines either in the incidence of tumors produced or in the individual tumor latent periods. Tumor latent periods were longer than those exhibited by cells of the high sarcoma-producing line (*3*). At higher inoculum levels, in excess of 5 million cells per mouse, the variability in tumor latent periods was reduced and the frequency of takes increased. In contrast to our experience with other tissue-culture cell strains (*2*, *21*) irradiating the mice before cell injection did not substantially alter the tumor latent periods, though the frequency of takes was slightly increased.

After 3 years of growth *in vitro* (43–52 months), the incidence of sarcomas from line 1745 cells was significantly reduced and was lower than that produced by line 2050 cells. However, the latent periods for develop-



TEXT-FIGURE 2.—Results of injecting cells of lines 1745 and 2050 into nonirradiated and X-irradiated mice of strain C3H after the cells had been cultured for 10 to 31 months and 43 to 52 months *in vitro*. *Top panel* shows percentage of mice developing sarcomas plotted against mean inoculum received by mice in the group. Number in parentheses indicates number of mice receiving injections. Results of assays in irradiated mice are shown by *open triangles*. *Lower panel* shows individual tumor latent periods plotted against inoculum size. Values for irradiated mice are indicated by *open circles*.

ment of tumors from line 2050 cells were prolonged, exceeding 5 weeks in both irradiated and nonirradiated hosts.

In summary, a decline in sarcoma-producing capacity was demonstrated to occur within the clone of tumor cells. The derivative lines differed in incidence of takes, and both lines differed from the parental high sarcoma-producing line with respect to tumor latent periods.

Chromosome Number and Morphology

The chromosomes of cell lines 1745 and 2050 were examined in January and March, 1958, respectively. The results of this examination are summarized in table 1. Both lines of cultured cells showed alterations from the normal mouse number and type. Line 2050 cells exhibited a modal number of 40 chromosomes with wide variations in the hyperdiploid range and a modal number of 10 metacentric chromosomes per cell. Mouse cells normally contain 40 acrocentric chromosomes. Line 1745, on which data are limited, contained some cells with fewer than the diploid number. Cells of this line exhibited a modal number of 5 metacentric chromosomes per cell. A comparison of these data with those for the parental high sarcoma-producing line and its derivatives (*16*) show, in general, a lower chromosome number with significantly fewer polyploid cells. The earlier chromosome studies were made 6 to 7 years after the tumor-cell clone had been separated from the high sarcoma-producing line.

TABLE 1.—Chromosome number and morphology in lines 1745 and 2050

Cell line	Chromosome No.																						
	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57
1745	1	2		3	2	4		2	2	2	2													
2050							14	5	11	5	3	2	1	1	2							1		

Cell line	Chromosome No.									Range of meta-centrics per cell	Modal No. of meta-centrics per cell	Number of cells analyzed
	58	59	60	61	62	63	64	65	121–130			
1745		1		2			1	2	2	1–7	5	28
2050		1	1		1				2	4–13	10	50

Arginase Activity of Cell Lines

When first examined for arginase activity in November, 1956, cells of line 1745 showed an activity 20 times and line 2050 cells 400 times the arginase activity of the high sarcoma-producing line 1742 (*1*). Activity in cells of the latter line was barely detectable. A question considered in the present study was whether the two cell lines would still differ in arginase activity when grown in an entirely different environment, such as that *in vivo*. Tumors produced by injecting cells of the two lines and

their derivatives into strain C3H mice were therefore examined for arginase activity. The culture cells were injected between July, 1957, and July, 1958.

Analyses of first- and second-passage tumors, summarized in table 2, revealed a significant difference between the two lines, during both the first and second passages in mice. Differences were also observed between tumors from the two lines of cells cultured in serum-supplemented chemically defined medium (lines 3074 and 3069). Values were higher than in the earlier published data for cells *in vitro* (*1*) because of the manganese activation used here and not in the previous estimates. The considerable range in values for the tumors as compared with cells *in vitro* might result from variable amounts of blood or other tissues or of necrosis present in the tumors.

TABLE 2.—Arginase activity of tumors produced by culture cells injected into mice

Cell line	Generation 1			Generation 2		
	Number of tumors	Arginase activity*		Number of tumors	Arginase activity*	
		Average	Range		Average	Range
1745	4	140	33–250	9	186	30–445
3074	1	125	125	—	—	—
2050	3	1093	860–6200	7	2761	1050–6200
3069	1	7100	7100	1	2150	2150

*Mg urea produced/min /100 g cell protein.

A second question considered was whether cells of either of the morphologically distinct clones (3423 and 3610) isolated from the low arginase line (3074) would differ from the parental line with respect to arginase activity. Assays of these cells (table 3) showed arginase activity in both clones somewhat higher than in the parental line 3074, but less than in cells of line 3069. The cells of both lines and clones cultured in the serum-supplemented chemically defined medium showed greater variability and an even larger difference between the two lines (3074 and 3069) than in the previous assays of cells cultured in the horse-serum, embryo-extract medium (*1*).

TABLE 3.—Arginase activity of culture cells

Date of assay	Arginase activity*			
	Line 3074	Clone 3423	Clone 3610	Line 3069
June, 1959	1	23	—	950
July, 1959	3	910	—	1230
August, 1959	10	12	—	1400
March, 1960	—	—	176	—

*Mg urea produced/min /100 g cell protein.

Further Biochemical Characterization of Lines 1745 and 2050

In this section, we will summarize additional biochemical properties of these cell lines which distinguish them from some other C3H mouse cells isolated from the same or different types of tissues and grown for long periods *in vitro* under similar culture conditions.

Glycogen synthesis and storage.—Cells of the two lines were compared with cells of three other lines or strains with respect to glycogen synthesis and storage. The three lines or strains were: 1) NCTC 1742, the high tumor-producing line referred to earlier; 2) NCTC 2049, a low tumor-producing line from the same cell as 1742 (*2, 3*); and 3) NCTC 721, a strain of cells from C3H mouse liver (*22*). All cells were grown in the horse-serum, embryo-extract culture medium.

As shown in table 4, only the cells of strain 721 from the liver synthesized and stored any detectable amount of glycogen. Cells of the liver strain contained an average of 300 mg glycogen per 100 g cell protein, whereas the 4 lines of connective-tissue or sarcoma origin contained no detectable glycogen when assayed 24 hours after a fluid renewal.

Comparison of cell lines with respect to certain other enzymes.—Cells of the two lines 1745 and 2050 and the parental high sarcoma-producing line 1742, as well as a derivative line and clone, 3069 and 2472, respectively, were assayed for activity of the enzyme β-glucuronidase. As indicated in table 5, activity of β-glucuronidase was low in the parental line 1742 and also in the derivative clone 2472, which was grown in a different medium and assayed 2 years later. However, activity was very high in both lines 1745 and 2050, approximately 20 times the activity found

TABLE 4.—Glycogen in cells 24 hours* after culture-fluid renewal

Cell line or strain	Years *in vitro*	Number of cultures examined	Glycogen content (mg/100 g cell protein)
1745	6	8	0
2050	6	9	0
1742	8	6	0
2049	8	7	0
721	10	5	300

*Glycogen content of strain 721 liver cells varies with time after glucose renewal in the medium (*23*).

TABLE 5.—β-Glucuronidase activity of culture cells

Cell line	Date of assay	β-glucuronidase activity*
1742	October, 1958	60
2472	November, 1960	43
1745†	October, 1958	1230
2050	October, 1958	1130
3069	November, 1960	1110

*μg of phenolphthalein liberated in 60 min at 37° C per mg of total tissue nitrogen.

†A tumor produced by injecting cells of line 1745 into a C3H mouse was also assayed in October, 1958, and exhibited an activity of 620 μg.

in the parental line 1742. Activity of β-glucuronidase in a tumor from 1745 cells was somewhat lower, but still about 10 times higher than that of the parental line 1742. It may be noted from these data that the activities of arginase and β-glucuronidase varied independently in the two lines 1745 and 2050.

Tompkins [8] examined several culture cell strains for enzymes capable of reducing the 4,5 double bond of ring A unsaturated 3-ketosteroids. In normal liver this enzyme catalyzes an irreversible reaction with reduced triphosphopyridine nucleotide (TPNH) as a reducing agent. The assay depended on the use of paper chromatography to follow the disappearance of C^{14}-labeled steroid substrates.

Cell lines 3074 and 3069, grown in the serum-supplemented chemically defined medium, as well as cells from strain L adapted to growth in the unsupplemented medium (strain 2071) (*6*) were included in this study and showed no activity. A clone (1469) from the mouse liver strain (*24*) was the only one of the cell lines tested which showed significant activity, and this activity was low as compared with normal, freshly excised mouse liver.

DISCUSSION

These studies have revealed morphologic changes and further alterations in arginase activity and apparently also β-glucuronidase activity within a single-cell clone of mouse tumor cells. These alterations appear to be hereditary, persisting for generations of growth *in vitro* as well as during growth of the cells as tumors in mice. Some alterations in chromosome number and morphology were also observed. Since these cells originated from a highly inbred, presumably homozygous mouse, the effect of increased chromosome number would primarily affect gene dosage but not qualitatively alter the genome. The morphologic alterations in chromosomes suggest that fragmentation, inversion, possible deletions, or translocations may have occurred, introducing position effects or reduced gene dosage. Although these two effects of altered gene dosage or position effects might account for the changes in hereditary properties, other explanations seem more probable.

During embryologic differentiation, a quantitative distribution of enzymes occurs without apparent change in chromosome structure so that each adult tissue is characterized by a unique pattern of enzymes (*25*). In the culture environment, a less orderly aberrant differentiation may have taken place, possibly associated with the sorting out, loss, or functional inactivation of autonomous cytoplasmic components, or the segregation of substrates and interactants during rapid cell division (*26*, *27*).

The third and most likely possibility is that, during rapid proliferation of these cell populations, gene mutations influencing enzyme synthesis and also cell morphology have taken place. In organisms which reproduce

[8] Unpublished experiments by Dr. G. M. Tompkins of the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md.

sexually or exhibit other phenomena for the transfer and recombination of genetic material and are therefore suitable for genetic analysis, gene mutations affecting morphology or enzyme synthesis have been frequently reported. For example, the high level of β-glucuronidase in tissues of the liver, spleen, and kidney of certain inbred strains of mice as compared with the low tissue level in the C3H mouse appear to depend on the presence of a single dominant gene (*28*). Although the most commonly demonstrated effect of mutation on enzymes is apparent loss or reduction of enzyme activity, gain of an enzyme activity after mutation has been reported (*29*).

In the transformations described here, both cell lines showed some morphologic changes in common and increases in enzyme activities over those of the original high sarcoma-producing line. A possible interpretation of these observations is that early in the history of the tumor clone, soon after mouse passage and before segregation of the two cell lines, genic mutations influencing arginase and β-glucuronidase synthesis as well as cell morphology took place. New cell types became established in the cell population and gradually overgrew the original cell type. Heritable changes within a clone have been shown to occur relatively early during culture *in vitro* and to become stabilized thereafter in the cell population (*8*). Possibly adaptation of mammalian tissue cells to conditions in culture after removal from the organism favors such genetic mutability.

The role of the two enzymes, arginase and β-glucuronidase, in the metabolism of the cells and the question as to whether the increased enzyme activities conferred any selective advantage to these cell variants *in vitro* have not yet been clarified. Although arginase is the enzyme concerned with the hydrolytic conversion of arginine to urea and ornithine, Westfall and associates (*1*) have so far been unable to demonstrate significant urea formation by intact cells in another high-arginase strain of liver cells in culture with arginine supplied in the medium. In the present study, the difference between the two cell lines, when returned to the mouse, in incidence of takes and duration of latent periods for tumor formation suggests some difference in their growth potential *in vivo*. Whether this difference could be correlated with their divergent arginase activities is uncertain.

The decline in transplantability of the cells with time in culture probably resulted from the development of immunologic incompatibilities with the host as has been shown to occur in other long-term cell strains (*3*, *21*). However, the failure of whole-body irradiation of the host to alter significantly the transplantability of the cells suggests that potentialities for growth of the cells *in vivo* may also have changed during prolonged culture.

In comparing these cells with other strains of C3H mouse cells grown for long periods under similar culture conditions, we noted certain biochemical differences, which appear to arise from two different phenomena. Apparently cultured cells, like transplanted tumors (*25*), frequently retain, even during rapid cell proliferation, certain vestigial functions of the tissue from which the cells originated.

Several examples of this persistence of functional activity *in vitro* are described: Mouse melanoma cells after more than a year (9 transplant generations) of proliferation in culture still produced melanin both in culture and tumor growth in the mouse (*30*). Malignant mast cells in culture maintained their characteristic capacity to store and manufacture both 5-hydroxytryptamine and histamine (*31*). The C3HBA mammary adenocarcinoma, after 1½ years and 35 transplant generations of growth *in vitro*, reproduced in mice the epithelial, glandular structure of the original tumor and retained the high arginase activity characteristic of C3H mouse mammary carcinomas (*32*).

A strain of C3H mouse liver cells, presumably of parenchymal origin (*22*, *33*), was also examined in this laboratory for certain liver functions and enzymes (*1*, *23*). Parenchymal liver cells *in vivo* are the major storage site for glycogen, though it has been demonstrated in cells of the skin epidermis and muscle. The liver cell strain in culture, as well as a derived clone, retained for years the capacity to synthesize and store glycogen. Of 13 mouse cell strains, including the cells of the present study and others of connective-tissue or mammary-carcinoma origin, and one human cell strain, only the cells of the liver strain and clone and the strain of human epidermal cells exhibited this function. The glycogen content of the liver cells in culture was never as great as that of liver from normal fasting mice, and during mouse passage one line of liver cells apparently lost this function which was not regained on subsequent culture of the cells *in vitro*.

Strain C3H mouse liver characteristically shows a high arginase activity, higher than found in any other normal mouse tissue (*1*). Cells of the liver strain and clone also showed high activity of this enzyme, considerably higher than observed in 6 strains of mouse fibroblasts of connective-tissue origin, HeLa, or a strain of human epidermal cells. Arginase activity in the culture cells was about 75 percent of that found in mouse liver. High rhodanese activity and detectable activity of the steroid reductase mentioned herein further indicated some retention of the enzyme pattern characteristic of liver parenchymal cells.

Not only certain specific tissue functions but also strain specificities can apparently be retained even when the cells are cultured in protein-free chemically defined medium. C3H isoantigens were identified in strain L fibroblasts of C3H origin after 5 years of continuous culture in chemically defined medium without protein or other supplement and after 20 years of growth in a horse-serum, chick-embryo-extract culture medium.

Since these experiments by Dr. Ruth Merwin of this Institute have not been published, the details will be presented here. Identification of C3H isoantigens was carried out by the harderian-gland technique (*34*, *35*). In 2 experiments suspensions of strain L cells cultured in NCTC 109 and suspensions cultured in the horse-serum, embryo-extract medium were injected into a total of 5 and 4 test mice, respectively. The used, unfiltered culture fluids were injected into 10 mice. Each test mouse of strain BALB/c carried a nonvascularized homograft of harderian-gland

tissue transplanted from a C3H/HeN mouse. Each graft was placed in the subcutaneous tissues so that the large pigment cells could be observed microscopically through a transparent window. The nonvascularized homograft does not initiate immunity but, if an injection specifically of C3H cells is made, the host becomes immune and the graft disintegrates. Each mouse received a subcutaneous injection of 0.5 ml of concentrated cell suspension (approx. 10^7 cells per mouse) or 0.5 ml of the used fluid. Disintegration of the harderian cells occurred in only 1 of the 10 mice receiving the unfiltered fluids but in all 9 mice receiving cells by 13 to 14 days after injection, which indicated a positive test for C3H isoantigens in the culture cells.

A second phenomenon to account for morphologic, functional, or biochemical differences between cell strains has been described in this paper. Apparently unpredictable, marked changes in enzyme activity, cell function, or morphology can occur during culture, probably as a result of genic mutations, though other possible mechanisms of change cannot be eliminated at the present stage of our knowledge.

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PLATE 167

Line 1745

Representative areas of cultures. × 200

FIGURE 1.—Culture in 15th-transplant generation, photographed in March, 1953, 11 months after single-cell isolation. *Note* elongated, spindle-shaped cells characteristic of high sarcoma-producing line (*2*).

FIGURE 2.—Culture in 34th-transplant generation, photographed in August, 1953, 16 months after single-cell isolation. At this time variations in cell size and shape were observed.

FIGURE 3.—Culture in 213th-transplant generation, photographed in February, 1957, 58 months after single-cell isolation. *Note* preponderance of rounded cells with occasional elongated fibroblast-like or large cells.

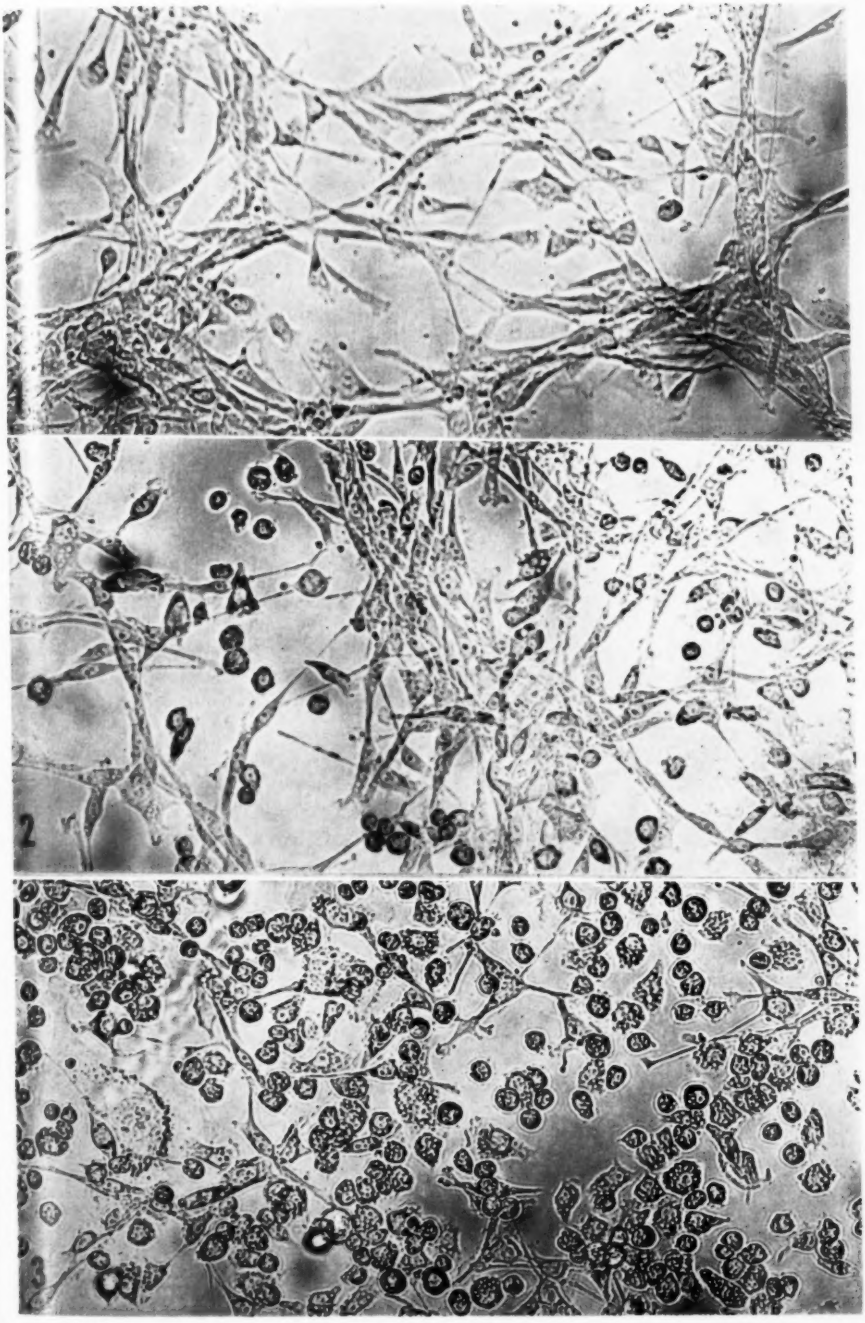

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PLATE 168

Line 2050

Representative areas of cultures. × 200

FIGURE 4.—Culture in 31st-transplant generation, photographed in August, 1953, 16 months after single-cell isolation. *Note* similarity to cells of strain 1745 in figure 1.

FIGURE 5.—Culture in 170th-transplant generation, photographed in April, 1956, 48 months after single-cell isolation. *Note* granular, rounded cells unlike elongated cells of figure 1. *Observe* two large polynuclear cells.

FIGURE 6.—Culture in 206th-transplant generation, photographed in February, 1957, 58 months after single-cell isolation. *Note* similarity to cells in figure 5.

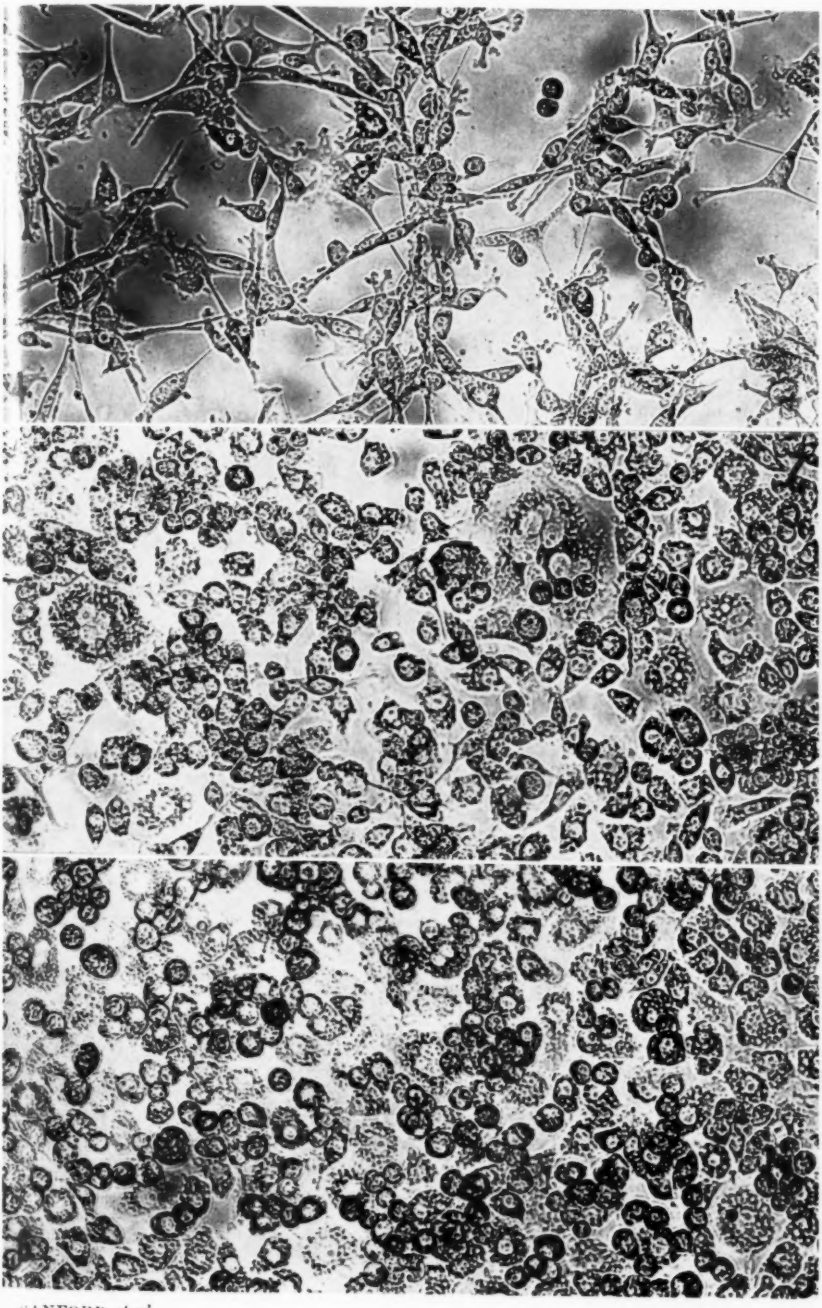


PLATE 169

Comparison between tumors produced by lines 1745 and 2050 and original tumor from which strains were derived. Hematoxylin and eosin. × 205

FIGURE 7.—Representative area of sarcoma from which clone of tumor cells was derived. *Note* fusiform cells arranged in bundles characterizing tumors from cells of high sarcoma-producing line (*2*).

FIGURE 8.—Area of sarcoma developed from cells of line 1745 injected in March, 1953, 11 months after single-cell isolation. *Note* similarity to figure 7.

FIGURE 9.—Area of sarcoma developed from cells of line 2050 injected in March, 1953, 11 months after single-cell isolation. *Note* similarity to figures 7 and 8.

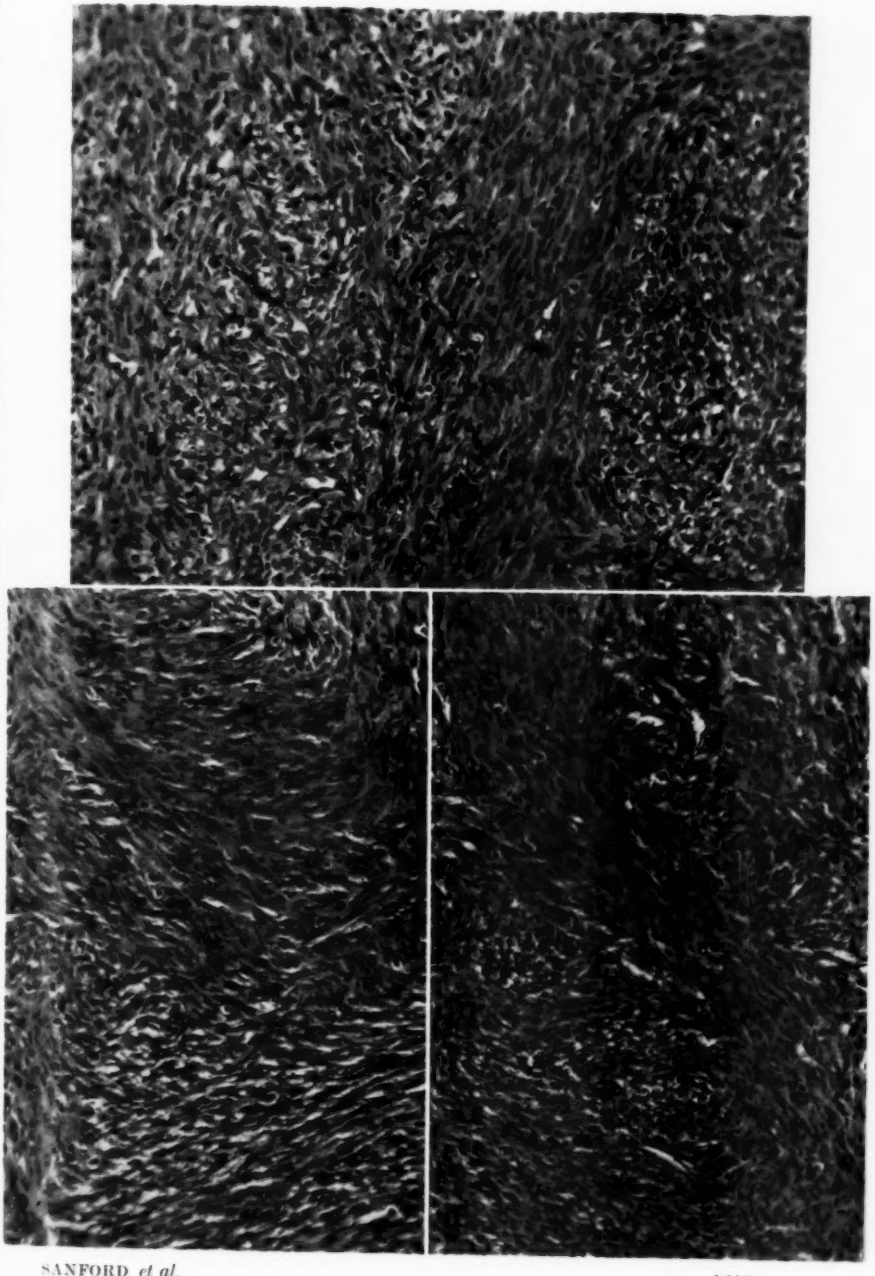


PLATE 170

Comparison of tumors produced by cells of the two lines injected into irradiated strain C3H mice. Hematoxylin and eosin. × 205

FIGURE 10.—Area of sarcoma developed from cells of line 1745 injected in July, 1956, 52 months after single-cell isolation.

FIGURE 11.—Area of sarcoma developed from cells of line 2050 injected in July, 1956, 52 months after single-cell isolation. *Note* distinctly rounder cells.

FIGURE 12.—Area of metastasis to liver from cells of line 1745. Cells were injected in February, 1958. *Note* rounder cells and giant cell in mitosis. Small portion of liver may be seen *upper left.*

FIGURE 13.—Area of lung showing metastasis from cells of line 3069. Cells were injected in July, 1958. *Note* similarity in cell morphology to figure 11.

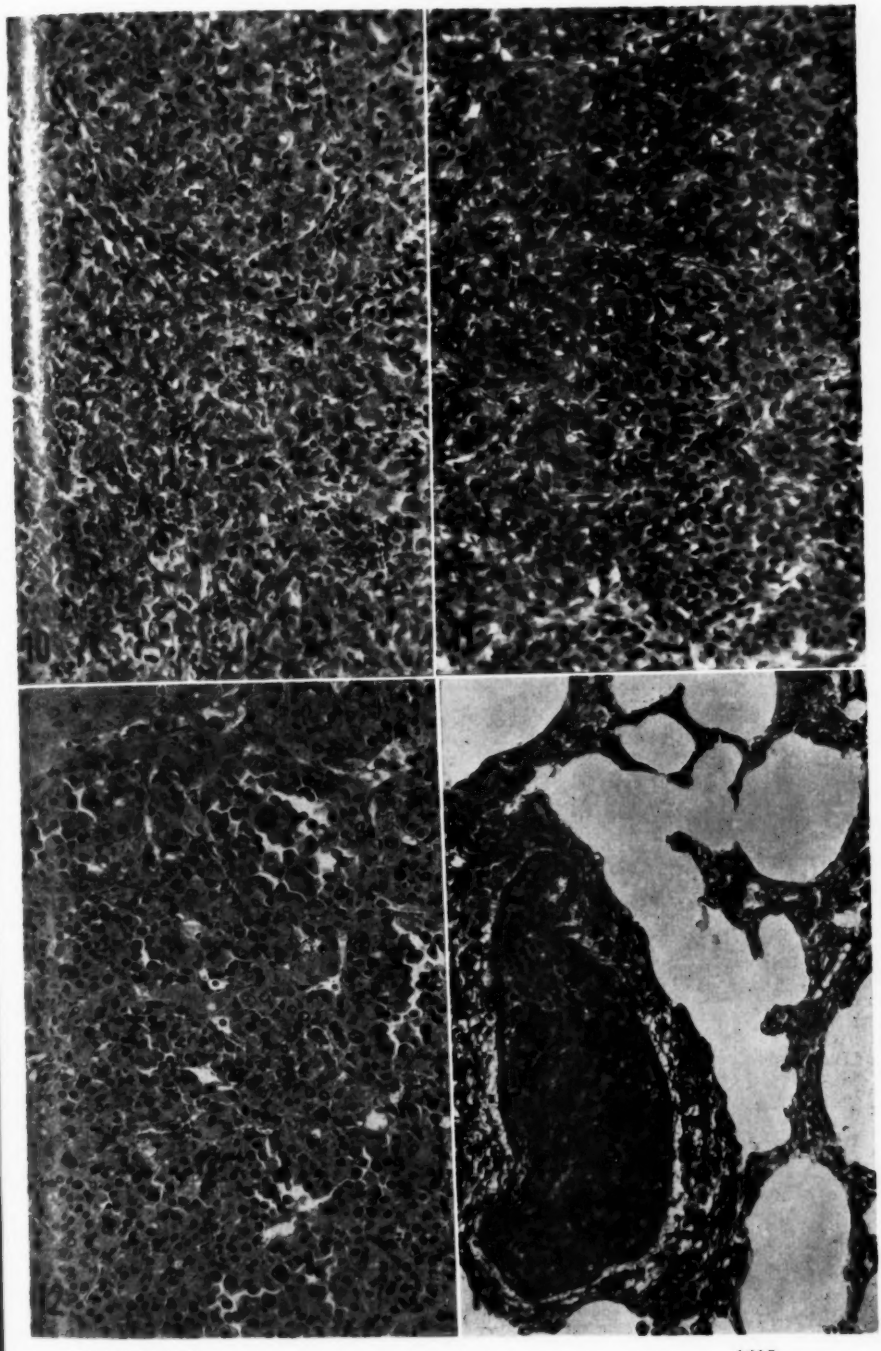


PLATE 171

Line 1745 derivatives

Representative areas of cultures. × 200

FIGURE 14.—Culture of clone 3610 showing peripheral outgrowth from capillary tube. Cells were photographed in January, 1959, 23 days after single-cell isolation. *Note* elongated, spindle-shaped cells characteristic of high sarcoma-producing line (*2*), and unusual circumferential pattern of growth rarely observed in culture strains.

FIGURE 15.—Culture of clone 3423 showing cells emerging from one end of capillary tube. Cells were photographed in May, 1959, 31 days after single-cell isolation. *Note* rounded cell morphology distinctly different from that of clone 3610. Cell migration rates also appeared reduced.

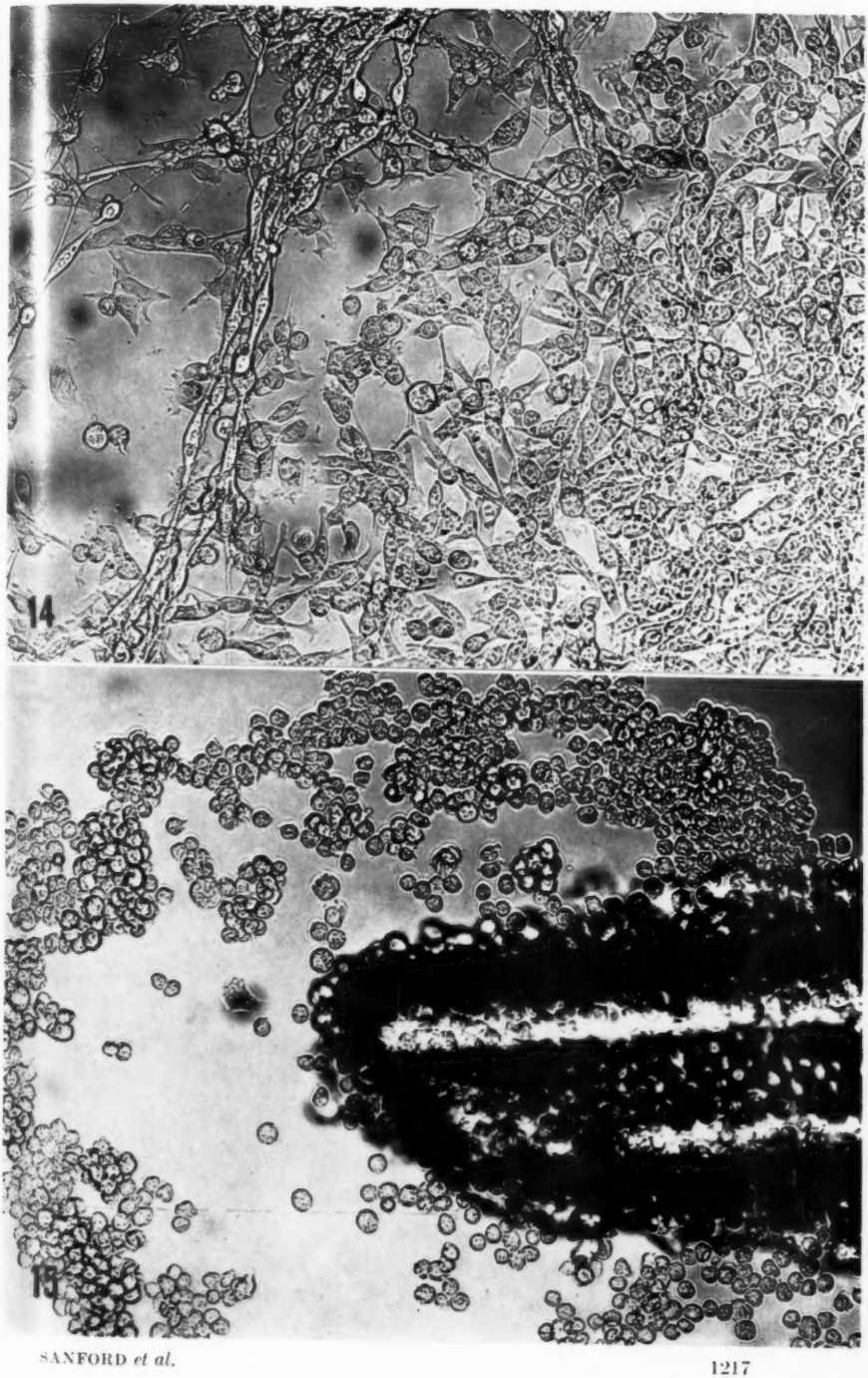

14
15

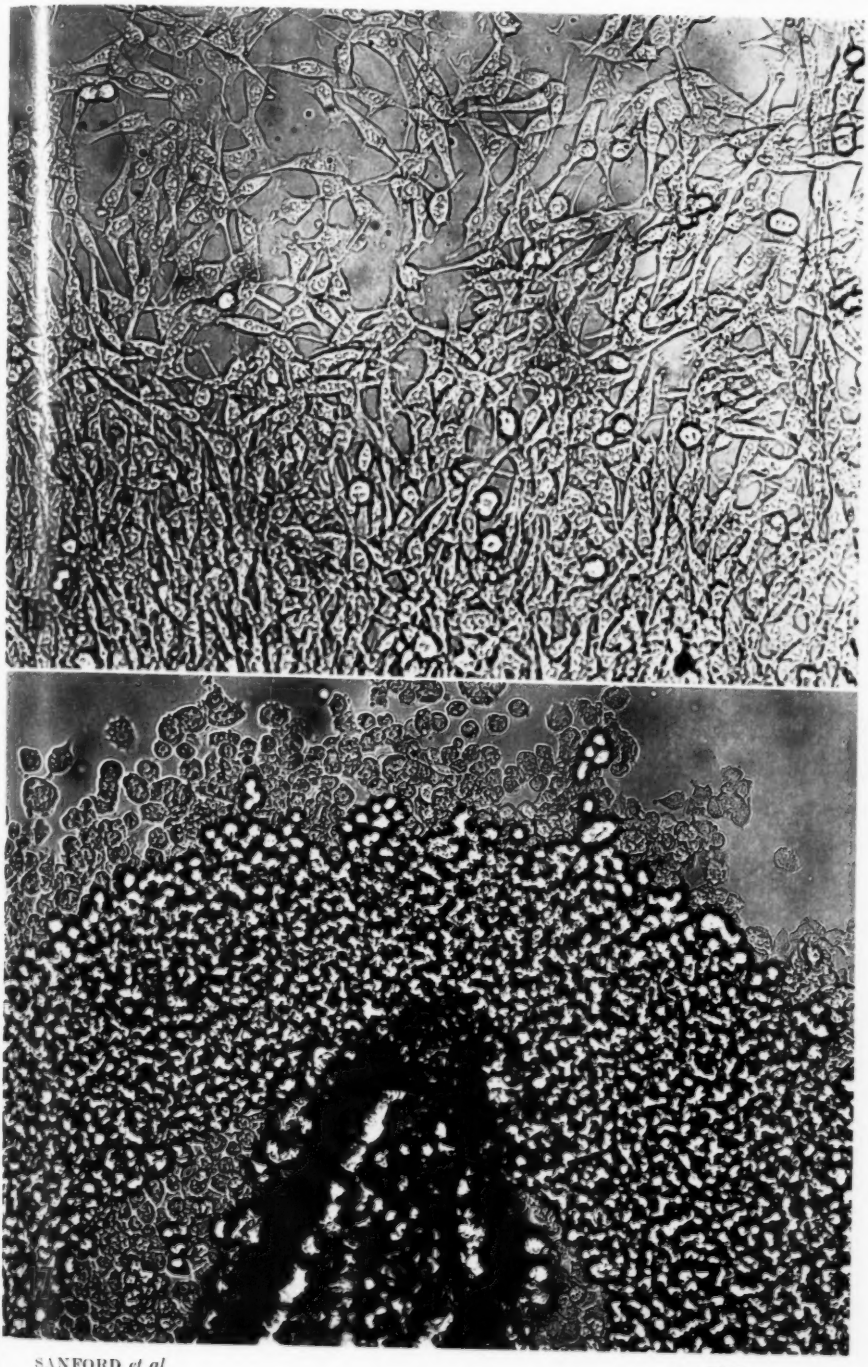
PLATE 172

Line 1745 derivatives

Representative areas of cultures. × 200

FIGURE 16.—Another area of clone 3610 photographed at same time as figure 14. *Observe* elongation of cells and their radial orientation from dense center of cell growth to *left*.

FIGURE 17.—Culture that originated from 3 cells of line 1745. Cells are emerging from end of capillary tube. *Note* resemblance to cells of clone 3423 and distinctive difference from those of clone 3610.



Some Ultrastructural Characteristics of a Series of Primary and Transplanted Plasma-Cell Tumors of the Mouse [1]

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SUMMARY

Cells of 21 plasma-cell neoplasms were examined with the electron microscope. Of these, 17 were mouse tumors, 11 of which were tested in transplant and found to be associated with serum hyperglobulinemia. Six primary tumors were also examined, 3 of which were studied in transplant. In addition, 4 multiple myelomas in man were examined. For comparative purposes other neoplasms from the same strains of mice, including 3 tumors diagnosed as undifferentiated plasma-cell tumors and 6 as fibrosarcomas, as well as normal plasma cells from lymph nodes and lamina propia of the gut, were examined. The cells of the plasma-cell tumors presented ultrastructural features basically similar to those of their normal prototypes. These features included 1) a well-developed Golgi complex with associated bodies having the ultrastructural characteristics of secretory granules; 2) a highly developed ergastoplasm occupying the remainder of the cytoplasmic area beyond the Golgi zone. The neoplastic plasma cells differed from normal plasma cells at the ultrastructural level in 3 respects: 1) The neoplastic cells did not show the great variation in size of the cisternae of the ergastoplasm characteristic of a series of normal plasma cells. 2) The neoplastic cells contained many more ribosomes lying free in the cytoplasm than did normal plasma cells. 3) Cells of all the plasma-cell neoplasms contained particles with the ultrastructural morphology of viruses (type A of Bernhard). No particles were observed in normal plasma cells or in multiple myeloma cells from 4 humans. The particles were not considered oncogenic viruses responsible for the development of plasma-cell neoplasms. A full discussion of this point is given.—J. Nat. Cancer Inst. 26: 1221–1267, 1961.

AT THE time this study was initiated no description of the ultrastructure of the cells of plasma-cell tumors was available, but the first evidence that cells of these tumors retained some degree of function was at hand (*1*). It was therefore decided to examine cells of the available tumors with the electron microscope and to determine whether there was a correlation between the structure and the specific serum hyperglobulinemia that

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characterizes generalized plasma-cell neoplasia of both mouse (*2*) and man (*3*). As the study progressed, more plasma-cell neoplasms became available, not only in transplant but as primary tumors. Also during this interval, an experimental procedure was developed which resulted in the induction of plasma-cell neoplasms in BALB/c mice (*4*).

As more transplantable neoplasms became available, greater differences in myeloma globulins were found. Further, it was determined that the characteristic myeloma-globulin production associated with each transplantable tumor was stable and heritable (*2*).

The observations in Waldenström's macroglobulinemia (*5*) imply that a correlation might be found if a morphologic comparison were made between cells producing macroglobulins and those producing myeloma proteins. In the present study no evidence was obtained regarding this particular point, but it was determined that the ultrastructure of normal plasma cells, characteristic of cells of synthesizing specific proteins, is mirrored, though imperfectly, in cells of plasma-cell tumors demonstrated to have retained functional activity. On the other hand, no variations in cell ultrastructure could be associated with specific aspects of function.

Because virus-like particles were seen in cells of all mouse plasma-cell tumors examined, two other groups of tumors were studied. The majority of these tumors, diagnosed either as undifferentiated plasma-cell tumors or as fibrosarcomas, arose in animals exposed to the same experimental procedure associated with the appearance of plasma-cell tumors in other animals.

Since, unfortunately, certain cellular components possessing a definite and characteristic ultrastructural morphology have had a confusing terminological history, the terms used in reference to these as well as to several other cell components are defined:

Ergastoplasm: the basophilic component of the cytoplasm which consists of endoplasmic reticulum and associated ribosomes (*6*).

Endoplasmic reticulum: an intracytoplasmic system of membranes which marginates cisternae within the ergastoplasm but extends to other parts of the cytoplasm as well (*7*). The membranes may be in continuity with the plasma membrane and inner cytoplasmic membrane and with membranes of the Golgi complex.

Ribosomes (*8*) (*ribonucleoprotein particles*): cytoplasmic particles, 150 to 250 A in diameter, with high electron-scattering power. The particles may lie free in the cytoplasm or may be in close association with the membranes of the endoplasmic reticulum. They are responsible for cytoplasmic basophilia.

Virus-like particles: During the last few years, particles with characteristic ultrastructure and not considered a normal constituent of protoplasm have been described in association with a variety of cells and, in some instances, have been causally related to virus diseases and to neoplasias. These particles vary in size from approximately 250 to over 1000 A in diameter and are not to be confused with ribosomes. When such particles are visualized in association with normal or neoplastic cells, only correlated electron-microscopic analysis and bioassays can determine whether the particles are the morphologic representation of a viral agent.

Golgi complex: a cytoplasmic system, usually located in the nuclear "hof," consisting of vacuoles, small vesicles, and layers of flattened sacs.

Nuclear membrane: the inner of two membranes located at the nuclear border. The outer of these two membranes is considered the inner boundary of the cytoplasm.

Secretory granules: granules of variable but usually relatively large size in the cytoplasm of many cell types known to secrete specific proteins. Characteristically they possess a homogeneous, moderately electron-dense matrix and are usually delimited by a single, thin membrane.

MATERIALS AND METHODS

Normal plasma cells for comparison with neoplastic plasma cells were obtained from lymph nodes of C3Hf/Lw mice killed without treatment, from a BALB/c mouse that received 34 horse-serum injections 3 times weekly plus skin painting with methylcholanthrene, and from a DBA/2 mouse that received 19 injections of horse serum 3 times weekly.

The mouse tumors examined are listed in column 1 of table 1. Column 4 indicates whether the tumor cells had at any time in their history been exposed to the mammary-tumor agent (MTA). A question mark in this column indicates that a question arose as to the possibility of mammary-tumor agent escaping into the peritoneal cavity through the pores or a break in the Millipore filter chamber previously inserted. Column 5 shows whether virus-like particles were observed. Column 6 indicates the type of serum myeloma protein found. A dash in this column means that no determinations were made. Column 7 gives a rough estimate of the relative number of bodies with ultrastructural characteristics of secretory granules present in the cells. The last column summarizes the experimental procedure used for animals in which the primary tumor arose and any features peculiar to the tumor.

On removal from animals, either under ether anesthesia or after being killed by cervical dislocation, tissues were minced with razor blades into very small pieces while immersed in an excess of chrome-osmium fixative, *p*H 7.4 (*9*) for 1 hour, postfixed for 1 hour in 10 percent neutral formalin, dehydrated in a graded series of ethyl alcohol (70–100%), and embedded in a mixture of 1 part methyl to 4 parts *n*-butyl methacrylate containing 0.25 percent azodi-iso-butyronitrile as an initiator (*10*). Embedding was carried out at 47° C. Sections were cut with a diamond knife on a Porter-Blum ultramicrotome, set to cut at 250 A, and then stained either with a saturated solution of lead acetate for 10 minutes, which was followed by exposure to the vapors of 5 percent NH_4OH, or with a half-saturated solution of monobasic lead acetate for 5 minutes (*11*). The electron micrographs were taken with a Siemens Elmiskop I electron microscope having a double condenser and a 50 μ objective aperture or with an RCA EMU-2C electron microscope having a 30 μ objective aperture.

RESULTS

The descriptions of normal and neoplastic cells that follow are the result of careful study of several hundreds of cell sections from each

TABLE 1.—Plasma-cell tumors

Tumor No. or name	Source	Primary or transplanted	MTA + or −	Particles + or −	Serum protein mobility	Secretory granules + or −	Sex	Strain	Experimental procedure and/or remarks
X5647	Pilgrim	T	+	+	β	+	F	C3H	Ovariectomy—partially differentiated cells present
X5563	"	T	+	+	γ	+	F	C3H	Ovariectomy—particles in Golgi zone and cisternae of ergastoplasm
MPC-1	Merwin	T	?	+	α_2	++	F	BALB/cAn	Millipore diffusion chamber containing C3H
MPC-2	"	T	?	+	$\beta + \gamma$	+	F	"	Mammary-tumor tissue, intraperitoneally and subcutaneously
MPC-3	"	T	?	+	β	+	F	"	Diethylstilbestrol pellet
MPC-4	"	T	?	+	α_2	+	F	"	"
Adj-PC-2	Potter	T	−	+	β	+−	F	"	0.3 Ml incomplete Freund's adjuvant + heat-killed staphylococcus, intraperitoneally and 20 MCA skin paintings
Adj-PC-3	"	P	−	+	—	+	F	"	
Adj-PC-5	"	P + T	−	+	γ	++	F	"	"
Adj-PC-6	"	P + T	−	+	$\beta + \gamma$	+	F	"	"
DPC-1	Dunn	T	+	+	$\beta + \alpha_2$	++	M	C3H/HeN	Whole AKR exorbital lacrymal gland, subcutaneously, particles in Golgi zone and ergastoplasm
SPC-1	Sanford	T	+	+	$\beta + \alpha_2$	+	M	"	C3H/He embryonic mince injected intraperitoneally; 425 r whole-body radiation
MPC-3′(*)	Merwin	T	?	+	—	+	F	BALB/c	Millipore diffusion chamber containing C3H mammary-tumor tissue, intraperitoneally and subcutaneously, diethylstilbestrol pellet—late-appearing MPC-3
H78950	"	P	−	+	—	+	F	"	Millipore filter, intraperitoneally, containing C3H MTA negative mammary-tumor cells
H86199 (MPC-10)	"	T	+	+	—	+	M	C3H/He	Millipore filter, intraperitoneally, containing plasma-cell-tumor cells
H75140 (MPC-9)	"	T	+	+	—	+	M	"	Millipore filter, intraperitoneally, containing S-37 tumor cells

H93595	"	P	?	+	—	+	F	BALB/c	Millipore filter, intraperitoneally, containing C3H MTA + mammary-tumor cells
H87733 (MPC-11)	"	P + T	?	+	—	+	F	"	Millipore filter, intraperitoneally, containing C3H MTA + mammary-tumor cells
Undifferentiated plasma-cell tumors									
70429	Dunn	T	+	+	—	—	F	C3H	Methylcholanthrene in tricaprolin
H78984	Merwin	P	+	+	—	—	M	C3H/He	Millipore diffusion chamber, intraperitoneally
H91061 (MPC-12)	"	T	?	+	—	—	M	BALB/c	" " " containing 70429 cells
H91530 (MPC-12)	"	T	?	+	—	—	M	"	Same as above, second generation
Fibrosarcomas									
H66591	Merwin	P	?	—	—	—	F	BALB/c	Millipore diffusion chamber containing MTA + mammary-tumor cells, intraperitoneally
H66590	"	P	?	—	—	—	F	"	"
H78927	"	P	?	—	—	—	F	"	"
H78987	"	P	?	—	—	—	F	"	"
H91461	"	P	+	—	—	—	F	"	"
H93652	"	P	—	+	—	—	F	"	Millipore diffusion chamber containing MTA—negative mammary-tumor tissue

*Primary tumor MPC-3 was inoculated by trocar into several mice. In 1 mouse the tumor grew rapidly and was ready for transfer in 2 months. This transplant was used to establish tumor MCP-3. In a second mouse the tumor did not become evident until 1 year from the date of implantation. The mouse was the source of tumor MPC-3'.

specimen, and the figures were selected as representative of an average of 50 electron micrographs in each instance.

Normal Plasma Cells

From previous work (*12*) and the work of others (*13–16*), the general features of the ultrastructure of typical plasma cells were already well known. For comparative purposes, however, plasma cells from lymph nodes of untreated mice, mice that had received repeated injections of foreign protein only, and mice that in addition had received several skin paintings with methylcholanthrene were studied. No essential differences were noted among the plasma cells from these 3 sources. They regularly possessed a large, well-defined Golgi zone in which bodies with the ultrastructure of secretory granules were frequently observed, as well as profiles of the usual flattened sacs and vesicles of the Golgi complex (fig. 1). Mitochondrial profiles were either oval (fig. 1) or filamentous (fig. 2) and prominent. The ergastoplasm was very conspicuous, occupying the remainder of the large cytoplasmic area. The cisternae within it, filled with a finely granular, moderately electron-dense substance and lined by membranes of the endoplasmic reticulum, varied considerably from cell to cell (figs. 1, 2, and 3). The majority of the ribosomes was associated with membranes—relatively few lying free in the cytoplasm (figs. 1, 2, 3, and 4). The nuclei of normal plasma cells frequently exhibited a relatively smooth contour with only occasional minor indentations (fig. 3). Rarely, they showed more marked indentations with margination and clumping of chromatin, the electron microscopic equivalent of the "cartwheel" nuclei characteristic of human plasma cells (fig. 4). Medium-sized and rather prominent nucleoli were a common occurrence (figs. 3 and 4).

Tumor Cells—General

The features by which neoplastic plasma cells could be distinguished from their normal counterparts were: at the light microscope level—their larger size and the greater lobulation of their nuclei; at the ultrastructural level—the relatively constant size of the cisternae of the ergastoplasm, a relative increase in the number of ribosomes lying free in the cytoplasm and not showing association with the membranes of the endoplasmic reticulum, particles with the ultrastructural morphology of viruses in the cisternae of the ergastoplasm and attached to the membranes of the endoplasmic reticulum. The particles occurred in cells of primary as well as transplanted tumors and were noted in primary tumors of mice which were free of the MTA. To determine whether the particles were a relatively specific feature of neoplastic plasma cells of the mouse, exocrine cells of the pancreases of mice carrying plasma-cell tumors Adj-PC-5 and -6 were examined for particles. None was observed.

Features common to both normal and neoplastic plasma cells were a large, well-developed Golgi complex and the presence of bodies with the

ultrastructural characteristics of secretory granules in the Golgi zone. Both had a large amount of highly organized ergastoplasm.

The cells of tumors diagnosed either as undifferentiated plasma-cell tumors or as fibrosarcomas were readily distinguished by their ultrastructure from both normal and neoplastic plasma cells. The primary difference was the small amount of endoplasmic reticulum in the cells of these tumors. The undifferentiated plasma-cell tumors could be distinguished from the others by the fact that their cells had many more ribosomes lying free in the cytoplasm. Virus-like particles were present in or associated with the cells of some of these tumors, but never was their ultrastructural morphology similar to that of the particles present in neoplastic plasma cells.

Plasma-Cell Tumors—Detail

As mentioned, cells of all the plasma-cell tumors examined contained virus-like cytoplasmic particles. These particles were of two types: A-1 which was in all plasma-cell tumors, and A-2, which appeared in tumors X5563 and DPC-1. Plasma-cell tumor MPC-1 was chosen to demonstrate the method of formation of the type A-1 particle. In the cells of this tumor, particles were seen lying free in the cisternae of the ergastoplasm and also apparently budding from the membranes of the endoplasmic reticulum (fig. 5). Usually the budding process appeared at random throughout the ergastoplasm, but occasionally a localized area in which numerous particles were caught in the process of budding was observed (fig. 6). Ribosomes, typically aligned along the membranes of the endoplasmic reticulum, were absent from the areas immediately surrounding the point of particle formation (figs. 5 and 6). The particles were all of the "A" type of Bernhard (*17*), consisting of two concentric membranes surrounding an electron-lucent center. Occasionally, clearly elongated forms were seen (fig. 5), and, rarely, doublets with separate inner membranes and common outer membranes were observed (fig. 6). The large majority of particles had circular or slightly ovoid outlines averaging 70 mμ in diameter. The outer membrane, which was less clearly defined than the inner, was approximately 5 mμ thick and was separated from the inner membrane by an electron-lucent space approximately 6 mμ wide. The inner membrane, which stained more heavily with lead subacetate, was also approximately 5 mμ wide. It delimited a central, relatively electron-lucent area slightly less than 30 mμ in diameter (fig. 14). This type particle was described recently in a transplantable fibrosarcoma induced by methylcholanthrene (*18*).

In the cells of tumors X5647 and MPC-3 a difference in distribution of the particles was observed. In the cells of tumor X5647 individual particles could be seen attached to various parts of the endoplasmic reticulum, but, as occasionally occurred in cells of MPC-1, it was more common to find concentrations of particles associated with a circumscribed area of the endoplasmic reticulum (fig. 7). Particles in the cells

of MPC-3 were moderate in number and also usually concentrated within circumscribed areas of the endoplasmic reticulum (fig. 8). In the cells of the second, later-appearing line of MPC-3, MPC-3′ (*see* footnote to table 1), particles were extremely numerous, filling many of the cisternae of the ergastoplasm (fig. 9) and occurring in the space between the nuclear and inner cytoplasmic membrane (fig. 10). They were occasionally attached to the inner cytoplasmic membrane (fig. 9) and to the nuclear membrane (fig. 11).

A variant on the site of particle formation was noted in tumors Adj-PC-5 and SPC-1. A prominent feature of these tumor cells was the presence of relatively large areas of endoplasmic reticulum, which formed numerous contiguous vesicles free of ribosomes (fig. 12). Particles were seen in the lumina and attached to the membranes forming these vesicles (figs. 12 and 13).

A variant on the mode of particle formation was that in tumors MPC-2 and H86199. In the cells of these tumors the membranes of the endoplasmic reticulum were thickened and exhibited greater staining with lead subacetate at the points of attachment of particles (fig. 14).

While type A-1 particles were present in tumor X5563 (fig. 15), type A-2 were more prominent and numerous. They have been described previously in this tumor by Howatson and McCulloch (*19*) and in other cell types by different investigators (*17*). These particles were concentrated in the circumscribed area near the nucleus, usually associated with but not actually within the Golgi complex (fig. 15). Occasionally they were seen just beneath the plasma membrane (fig. 16). They apparently form in the continuous cytoplasmic phase outside the cisternae of the ergastoplasm. These particles show direct continuity with elongated, irregularly shaped bodies, which sometimes possess a single-limiting and at other times a double-limiting membrane (fig. 17). While having approximately the same outer diameter as the type A-1 particles, averaging 75 mμ, and consisting of two concentric membranes separated by an electron-lucent space, these components and the central area which they surround all had different dimensions. The total width of the two membranes plus the electron-lucent space separating them averaged 15.5 mμ in the particles first described. In type A-2 particles the width of these components was approximately 12 mμ and the central space which they surround was approximately 50 mμ, rather than the 30 mμ average for type A-1 particles (fig. 14).

These two types of particles, in about the same proportion, were seen in DPC-1. Many of the cells of this tumor had a less prominent Golgi complex than the cells of the other tumors in this series, but in some cells the complex was expanded, and related to it were bodies with the ultrastructural characteristics of secretory granules as well as particles identical to the type A-2 observed in the cells of tumor X5563 (fig. 18). The type A-1 particle common to all other plasma-cell tumors was seen in the cisternae of the ergastoplasm (fig. 18). In this tumor, the ergastoplasm had an organization typical of plasma cells, except that a secondary in-

folding of small strands or cords of ergastoplasm into the larger cisternae was a common occurrence (fig. 19).

Other features peculiar to, or especially prominent in, the cells of particular tumors of this series were: 1) the large number of bodies with the ultrastructure of secretory granules associated with the Golgi complex of cells of tumors DPC-1 and Adj-PC-5 (fig. 20); 2) the direct continuity between mitochondria and single-membrane-bound vesicles containing numerous small granules in cells of tumor MPC-3′ (fig. 21); 3) the large areas of cytoplasm devoid of endoplasmic reticulum in some of the cells of tumor X5647. This tumor was the one exception to the observation that the variation in size of the cisternae of the ergastoplasm from cell to cell was less in neoplastic plasma cells than in normal plasma cells (fig. 22).

Multiple Myeloma

Since type A-1 particles were in cells of all the plasma-cell tumors of mice studied, the cells of a series of 4 human multiple myelomas were examined with the electron microscope and possessed the same basic ultrastructure as the cells of plasma-cell tumors of the mouse. The Golgi complex was well developed, occupying a large juxtanuclear area in which one (fig. 23) or two (fig. 24) prominent centrioles were frequent. The ergastoplasm was also highly developed, occupying the remainder of the cytoplasmic area (fig. 25). Many of the cells had numerous narrow, branching processes extending for short distances into the surrounding medium (fig. 25). No type "A" or any other virus-like particles were observed in any cells examined.

Undifferentiated Plasma-Cell Tumors

The group of 3 neoplasms diagnosed as undifferentiated plasma-cell tumors was examined to compare the ultrastructure of their cells with that of cells of typical plasma-cell tumors. A further reason for interest in 2 of these tumors and in the 6 tumors diagnosed as fibrosarcomas was that they arose in mice exposed to the same experimental procedure (intraperitoneal insertion of a Millipore filter chamber containing, in most cases, mammary-tumor cells) which had in other mice been followed by the appearance of plasma-cell tumors.

The cells of the ascites form of tumor 70429 presented little evidence at the ultrastructural level to suggest plasma-cell origin (*20*). The ergastoplasm was poorly developed, consisting of a few membranes of the endoplasmic reticulum with associated ribosomes. The spaces between the membranes were seldom dilated into cisternae and the majority of the numerous ribosomes lay free in the cytoplasm (fig. 26). The Golgi complex, consisting of a few flattened sacs and vesicles (fig. 27) was not prominent. Unusual cytoplasmic inclusions were seen in some cells (fig. 28) and occasionally type "A" particles were observed extracellularly (fig. 29).

In the cells of tumor H91061, which arose in a mouse in which the inserted Millipore filter chamber contained cells of tumor 70429, the cytoplasm again contained but small amounts of ergastoplasm (fig. 30). The free ribosomes appeared in large enough numbers to result in prominent cytoplasmic basophilia. In this case, particles with the ultrastructural morphology of viruses were both in an intracellular (fig. 30) and in an extracellular position (fig. 31). There was evidence of particle formation, both at the plasma membrane (fig. 32) and in association with the endoplasmic reticulum. The intracellular particles were approximately 78 mμ in diameter. The extracellular particles averaged 90 mμ in diameter, and since electron-dense nucleoids staining heavily with lead subacetate have been seen in some of them, they have been classified as type C particles (*17*).

Sarcomas

Most of the cells of the fibrosarcomas were basically similar to one another in ultrastructure. They possessed somewhat more ergastoplasm than the cells of the undifferentiated plasma-cell tumors (fig. 33). Again, however, they were readily distinguished from neoplastic plasma cells. In the cells of only one of these tumors, H93652, were virus-like particles observed. In some cells of this tumor there were occasional type "A" particles within small smooth vesicles, presumably modified endoplasmic reticulum. The largest of these particles measured approximately 90 mμ in diameter (fig. 34).

DISCUSSION

A. Cytoplasmic particles.—Seventeen different primary and transplanted plasma-cell neoplasms of the mouse were examined with the electron microscope. All 17 (18 transplant lines) contained cytoplasmic particles (type A-1) that resembled certain known viruses by possessing a double-membrane outer shell. These particles consistently lacked a central nucleoid.

This unequivocal correlation of neoplastic plasma cells and virus-like particles raises two distinct questions which are discussed in relation to other established facts regarding oncogenic and other viruses.

The first and relatively complex question relates to epidemiology. The material itself is indicative of the complexity of the problem since it is necessary to divide the 17 neoplasms into 3 groups for this analysis.

The first group consists of: *a*) C3H/He mice with ileocecal plasma-cell neoplasms (X5563, X5647, DPC-1, and SPC-1), and *b*) C3H/He mice with Millipore-diffusion-chamber-evoked plasma-cell neoplasms (H86199 and H75140). C3H/He mice contain the MTA and therefore carry a known tumor virus.

The second group consists of BALB/cAn mice with Millipore-diffusion-chamber-evoked plasma-cell neoplasms (MPC-1, -2, -3, and -4, H93595 and H87733). These chambers contained mammary-tumor cells positive

for MTA and the possibility exists that the MTA may have escaped from the chambers.

The third group consists of: *a*) BALB/cAn mice with adjuvant-staphylococcal-emulsion-evoked plasma-cell neoplasms (Adj-PC-2, -3, -5, and -6), and *b*) a Millipore-diffusion-chamber-evoked plasma-cell neoplasm (H78950). Strain BALB/cAn mice do not carry the MTA though they are highly susceptible to it. The tumors in group 3 were at no time exposed to the MTA and therefore the MTA can be eliminated as the source of the type A-1 cytoplasmic particles. This cannot be claimed for the type A-2 particles, however, since this particle was only in tumors that arose in C3H/He mice carrying the MTA.

Other known viruses which may conceivably be carried by strain BALB/c mice are LCM (lymphocytic choriomeningitis) virus, K virus, SE polyoma virus, Moloney virus, and the Friend virus. Particles described in association with the presence of the K, polyoma, Moloney, and Friend viruses are morphologically distinct from those described here and may thus be eliminated. Unpublished studies in which the lymphatic organs and choroid plexuses of a large number of mice infected with LCM were examined with the electron microscope by Dalton did not reveal the presence of type A-1 particles in any of the material. Further, mice bearing transplants of the plasma-cell neoplasms do not become ill, and it is known that transplanted tumors harboring LCM will infect susceptible hosts.

A second question is that of the significance of these particles in relation to oncogenesis. Several years ago, when these particles were first noted in cells of plasma-cell tumors, there were reasonably good *a priori* reasons for considering the possibility that they might represent an inciting agent. Friedlaender and Moore (*21*), Adams and Prince (*22*), and Yasuzumi and coworkers (*23–25*) all described particles in Ehrlich ascites cells as somewhat similar in form, location, and mode of origin to the type A-1 plasma-cell tumor particles. Possibly correlated with these observations was the demonstration by Graffi (*26*) that a filterable agent capable of inducing leukemia in mice was present in the cells of the Ehrlich tumor. Comparable particles have been noted in Sarcoma 37 (*27*, *28*), and it was from Sarcoma 37 that Moloney obtained a cell-free extract which was demonstrated to contain a lymphoid-leukemia-inducing agent (*29*). However, the evidence to date does not support the view that the type A-1 particles represent either infectious oncogenic or passenger viruses:

1) Virus particles of the fowl leukosis complex (*30*, *31*) and the particles associated with the Friend (*32*) and Moloney (*33*) leukemia develop electron-dense nucleoids at maturation, while the type A-1 particles of plasma-cell neoplasms do not. The nucleoid is the anatomical structure of a virus assumed to contain the reproductive nucleic acid. Thus these particles would appear to be incomplete or noninfectious. Further, bioassays with cell-free extracts of tumor X5563 by Howatson (*34*), MPC-2 (*35*) and of MPC-3, and MPC-4 at this Institute have given negative results.

2) The particles have not been observed outside the cells. All other infectious viruses are seen outside as well as inside cells and this finding would be a prerequisite for infection, since the virus must be able to survive beyond its point of origin to propagate.

3) The particles are associated with, and arise from, the membranes of the endoplasmic reticulum. Examination of these membranes reveals pathologic changes in addition to particle formation. The membrane-ribosome relationship is disturbed and this disturbance takes several forms. First, the ribosomes were regularly absent from the membranes in the regions of attachment of particles. Second, in some instances, groups of small vesicles derived from the endoplasmic reticulum were present. These vesicles were free of ribosomes. Third, in other instances, membranes of the endoplasmic reticulum were thickened and showed greater affinity for lead subacetate in regions of attachment of particles. Fourth, the relative amount of endoplasmic reticulum is reduced from that characteristic of normal plasma cells and many more ribosomes lie free in the cytoplasm.

4) If these particles were viruses with a special predeliction for cisternae of the ergastoplasm, normal cells of the same host with a similar morphology might be expected to harbor the agent as well. Examination of exocrine cells of the pancreas in two cases were negative. Further, particles were not seen in normal plasma cells of strain BALB/c, C3H/He, or DBA/2 mice.

All this evidence suggests that the particles do not represent an agent responsible for the development of neoplastic plasma cells, but rather that the neoplastic plasma cell could well be a prerequisite for the appearance of the particles.

B. Protein synthesis and secretion.—The neoplastic plasma cells of the various tumors clearly have an abundance of the cytoplasmic apparatus associated with active protein synthesis (ergastoplasm). Of the 17 tumors, 11 were studied and shown to produce myeloma globulin. In one undifferentiated plasma-cell tumor, 70429, in which the cells had little ergastoplasm, no evidence of myeloma-globulin production could be demonstrated (*2*). From this evidence it seems reasonable to make a tentative correlation between retention of function and maintenance of plasma-cell morphology.

The question of whether myeloma globulins are disordered proteins (the consequence of neoplasia) or are normal proteins is difficult to answer. This is not the place to consider this question in detail, but there are several points in connection with it which should be noted. First, the characteristic myeloma-globulin formation by a plasma-cell tumor is stable and heritable. Second, the evidence to date, though incomplete, supports the concept that the myeloma globulins are structurally more normal than abnormal. In other words, they are proteins which are not new or unique to the species and genotype of the host. Third, the unusual situation of a cell geared to the synthesis of large quantities of specific protein, and at the same time required to produce the protein necessary

for the growth imposed by the presence of the neoplastic state, may be reflected in the modified ergastoplasm and the presence of the virus-like particles already described in detail.

While the sampling of human multiple-myeloma cells was relatively small compared with that of the cells of the mouse neoplasms, it would appear to be great enough to require an explanation for the differences observed in the two groups, especially since it confirms the findings of Braunsteiner (*36*). In contrast to the neoplastic plasma cells of the mouse, the only obvious differences noted between normal plasma cells and human multiple-myeloma cells was the presence of numerous, small, cell processes and prominent centrioles in the latter. It is possible that a larger sampling of a greater number of tumors might produce evidence which would provide a key to an understanding of these differences between the neoplastic plasma cells of mouse and man that is not presently available.

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All figures are electron micrographs of normal, stimulated, and neoplastic cells of the mouse or man. When but one micron marker is present on a plate, it is a reference for all figures on that plate. All tissues were fixed in chrome-osmium (*p*H 7.4).

Abbreviations

C = Centriole
CI = Cisternae of ergastoplasm
CM = Inner cytoplasmic membrane
E = Endoplasmic reticulum
ER = Ergastoplasm
G = Golgi vesicles
GC = Golgi complex
GM = Golgi membrane
M = Mitochondria
N = Nucleus
NM = Nuclear membrane
NP = Nuclear pore
NU = Nucleolus
P = Particle with viral ultrastructure
PM = Plasma membrane
R = Ribosomes
S = "Secretory granules"

PLATE 173

FIGURE 1.—Portion of plasma cell from lymph node of 5-month-old DBA/2 male mouse. Node drained a region into which horse serum was injected 19 times (3 times weekly). Margin of the nucleus is at *lower border* of figure, and *immediately above* is a well-defined Golgi zone with the usual membranes and vesicles. *Central* to the Golgi membranes is a group of bodies with the ultrastructural features of secretory granules. Enclosing the Golgi zone from *above* are several closely spaced layers of ergastoplasm. Oval and elongate profiles of mitochondria are also present. Lead subacetate stain. × 26,000

FIGURE 2.—Portion of another plasma cell from same material as that of figure 1. Again a prominent Golgi complex is present but in this instance the cisternae of the ergastoplasm are considerably larger than those of cell in figure 1. Filamentous mitochondrion is visible near *lower left* of figure. Lead subacetate stain. × 26,000

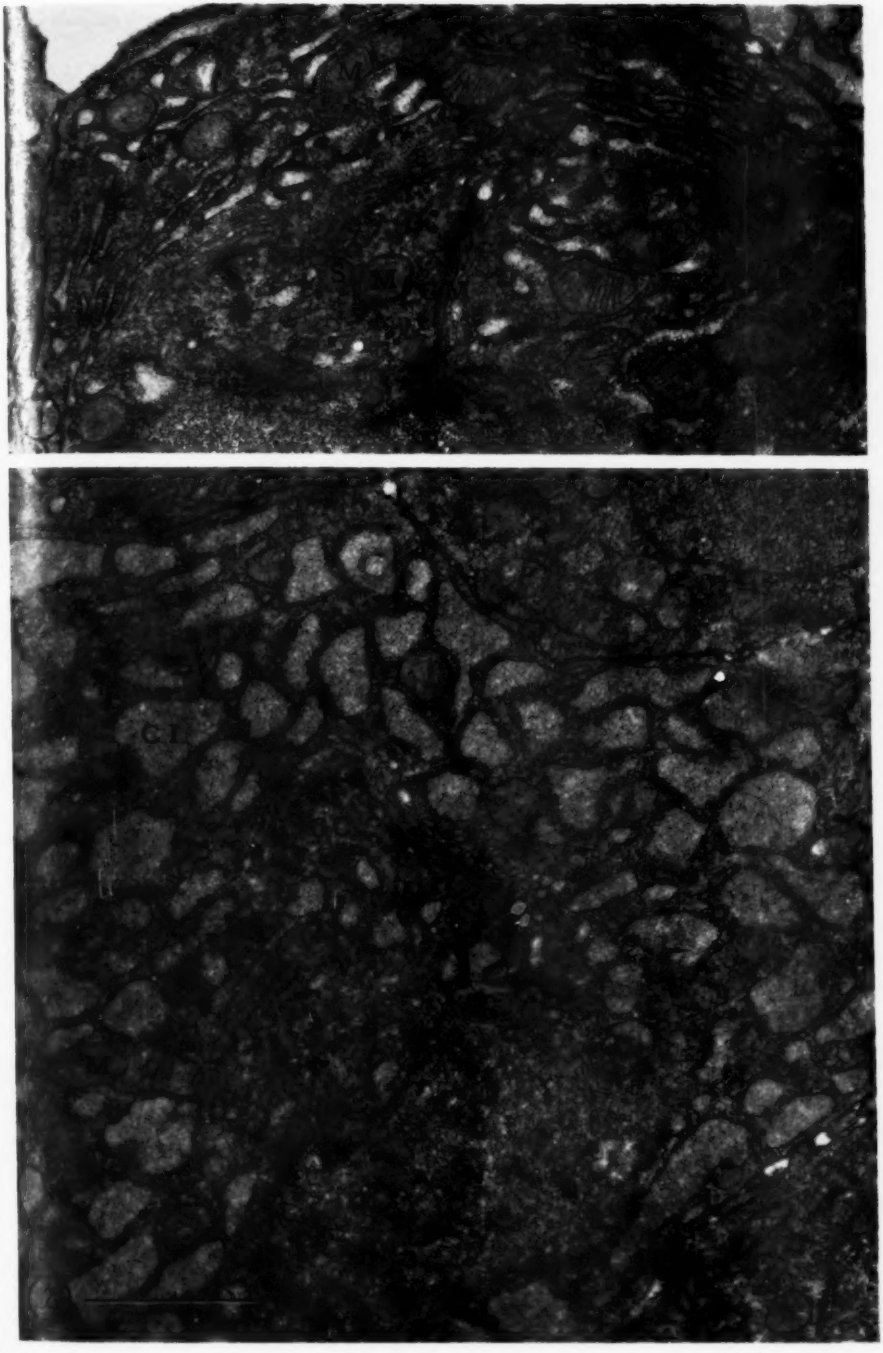

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PLATE 174

FIGURE 3.—Another plasma cell from same material as that of figure 1. Golgi zone is not visible. Cisternae of ergastoplasm show dilations intermediate between those in figures 1 and 2. Lead subacetate stain. × 35,000

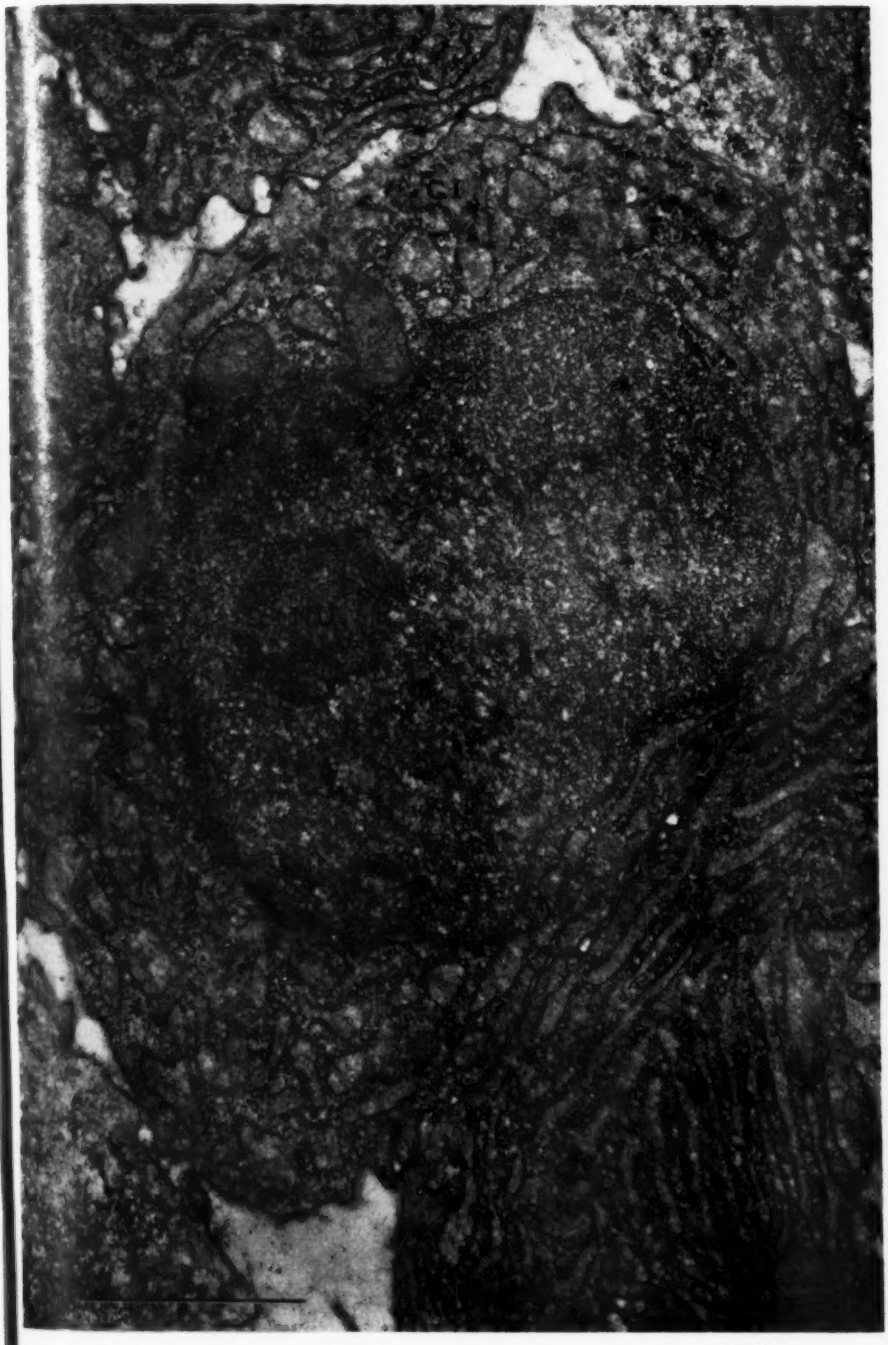
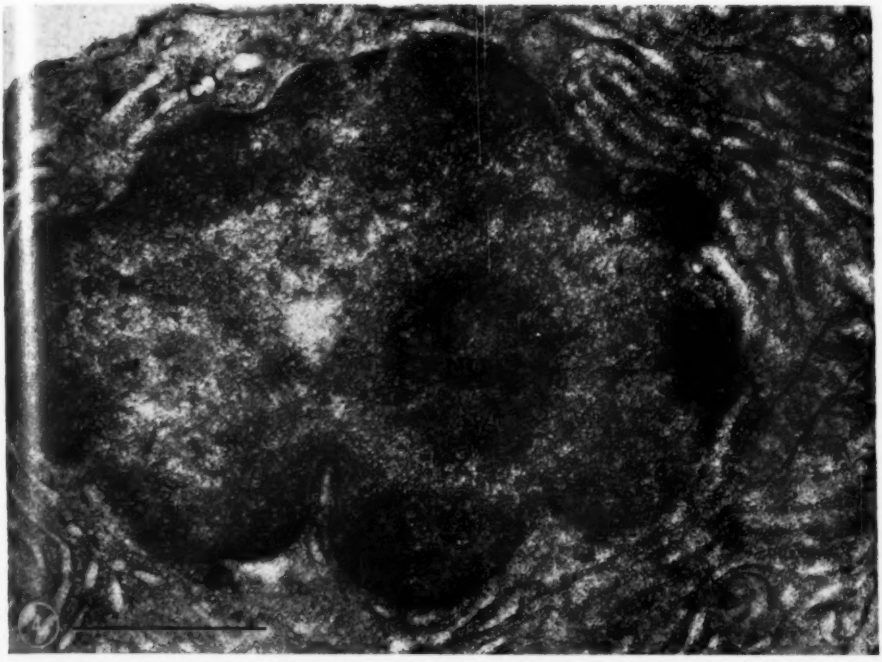


PLATE 175

FIGURE 4.—Portion of plasma cell from mediastinal lymph node of 20-month-old, untreated, C3Hf/Lw female mouse. Nuclear membrane shows several moderately deep indentations and chromatin appears condensed at various points along nuclear margin—the electron microscopic equivalent of "cartwheel" nucleus characteristic of human plasma cells. Cisternae of ergastoplasm are similar to those of cell in figure 1. Lead acetate stain followed by exposure to NH_4 vapor. × 30,000

Electron micrograph by R. F. Zeigel, N.C.I.

FIGURE 5.—Portions of two cells of plasma-cell tumor MPC-1. Toward *upper right* are plasma membranes of the two cells and near *bottom* of figure is nucleus of one cell with several nuclear pores. Particles with ultrastructural morphology of virus are present, some lying free in cisternae of ergastoplasm and some attached to membranes of endoplasmic reticulum that are devoid of ribosomes near areas of continuity with particles (*upper arrow*). Elongated form is at *A*. *Note* conspicuous small granules in the mitochondria (*lower arrow*). Lead subacetate stain. × 53,000



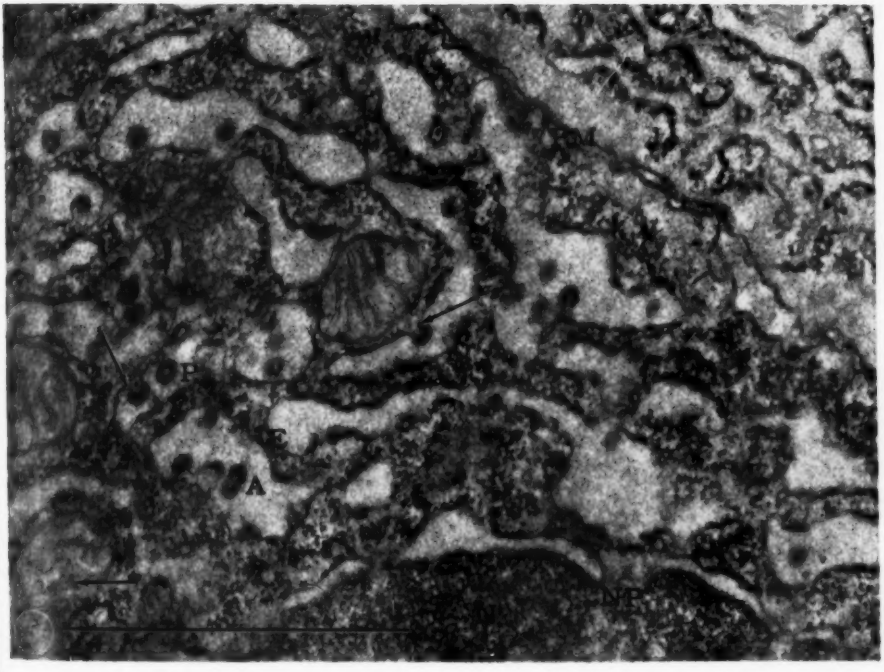

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PLATE 176

FIGURE 6.—Part of cytoplasm of cell from plasma-cell tumor MPC-1. In *lower part* of figure several particles lie free in cisternae of the ergastoplasm. A doublet with separate inner membranes and common outer membrane appears at *lower right* (*arrow*). Near *upper part* of figure many particles are concentrated in limited area. Several incomplete particles (*arrows*) are in continuity with the endoplasmic reticulum. *Note* at points of continuity the endoplasmic reticulum is free of ribosomes. Lead subacetate stain. × 53,000

FIGURE 7.—Part of cytoplasm of cell of plasma-cell tumor X5647. At *left* of *center* is group of particles, some free in cisternae of ergastoplasm, others attached to endoplasmic reticulum. This grouping or concentration of particles is characteristic in cells of this tumor. At a distance from this group are two particles, one attached to endoplasmic reticulum (*right arrow*), the other lying free in cisterna (*left arrow*). *Note* small granules in one mitochondrion. Lead subacetate stain. × 53,000

FIGURE 8.—Part of cytoplasm of plasma-cell tumor MPC-3. Several isolated particles (*arrows*) lie free in cisternae of ergastoplasm but majority is concentrated in one circumscribed area. This concentration of particles is as characteristic of cells of this tumor as it is of cells of tumor X5647. × 53,000

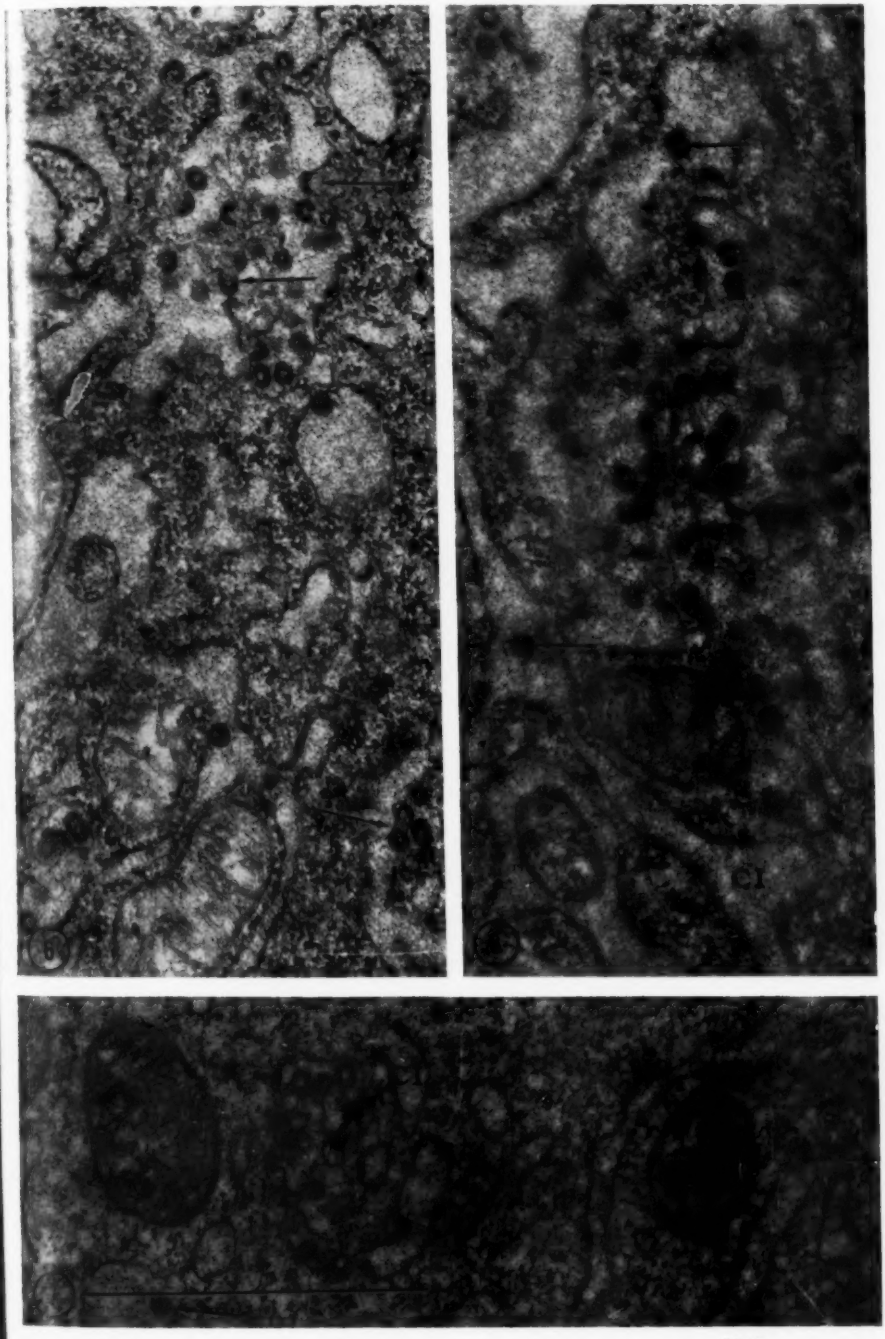
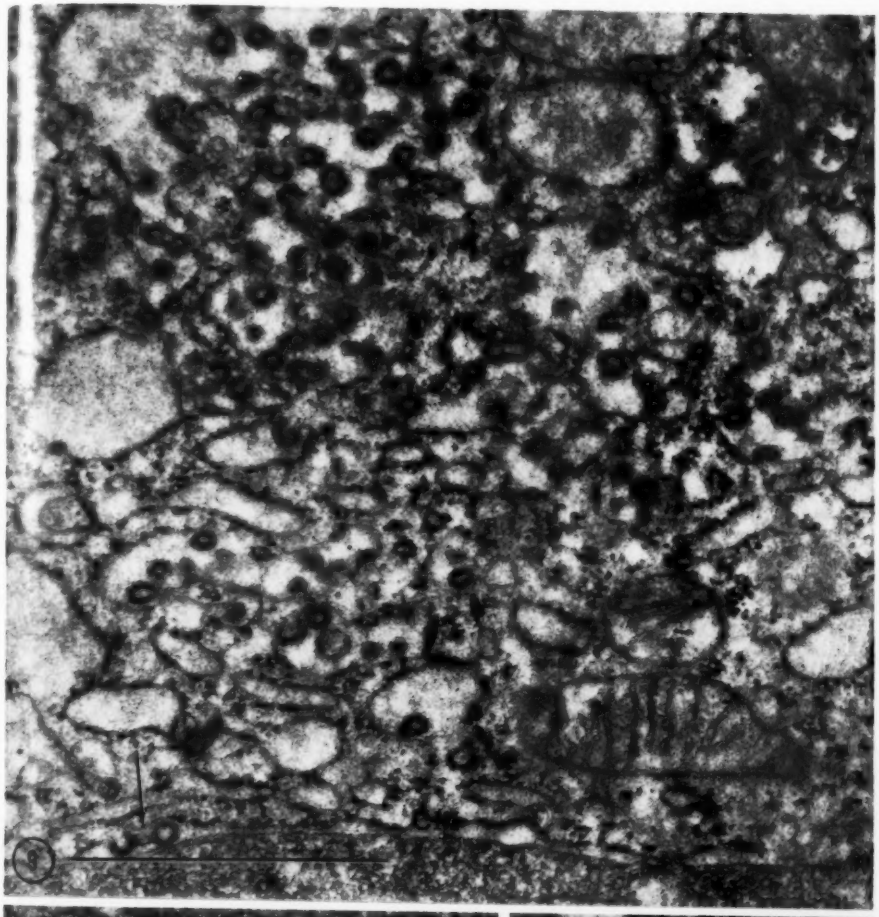


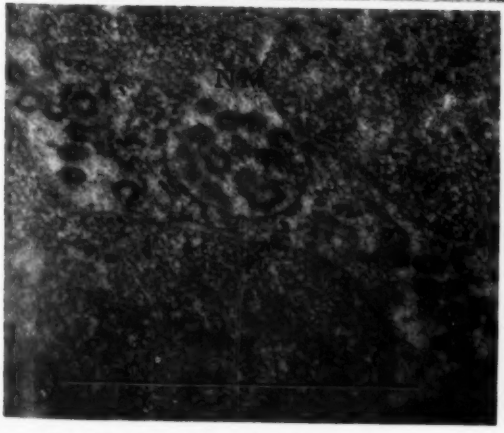
PLATE 177

FIGURE 9.—Part of cell of plasma-cell tumor MPC-3′. This tumor is first-generation transplant of tumor MPC-3 which appeared in mouse 1 year after implantation. Cells of MPC-3′ were characterized by greater number of particles than cells of any other plasma-cell tumors examined. Particles were frequently noted protruding from the inner cytoplasmic membrane (*arrow*). *Note* granules in mitochondrion near nuclear border. Lead subacetate stain. × 53,000

FIGURE 10.—Part of cell of plasma-cell tumor MPC-3′ showing deep fold in nucleus. Fold opens to cytoplasm toward *lower right* and ends beyond figure to *left*. Many particles lie free in space between the nuclear membrane and inner cytoplasmic membrane. Lead subacetate stain. × 53,000

FIGURE 11.—Part of another cell of plasma-cell tumor MPC-3′. Border of nucleus is to *left* and cytoplasm is to *right*. One particle lies free in space between nuclear membrane and inner cytoplasmic membrane. Other particle is attached to nuclear membrane (*arrow*). The marker represents 0.1 μ. Lead subacetate. × 53,000





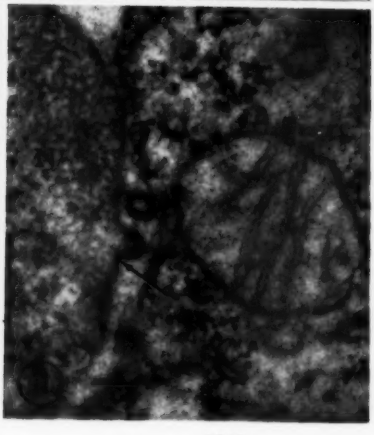


PLATE 178

FIGURE 12.—Area of cytoplasm of cell of plasma-cell tumor Adj-PC-5. From *center* to *left* are groups of small vesicles which are continuous with endoplasmic reticulum (*right arrow*). Particles are present within vesicles and attached to their walls (*left arrow*). Lead subacetate stain. × 53,000

FIGURE 13.—Another area of cytoplasm of cell of plasma-cell tumor Adj-PC-5 in which portion of Golgi complex is visible at *right*. To *left*, and separated from Golgi complex by narrow band of ergastoplasm, is a group of vesicles similar to those in figure 12. Several particles, two of them with incomplete walls, are present, each within separate vesicle. Lead subacetate stain. × 53,000

FIGURE 14.—Area of ergastoplasm in cell of plasma-cell tumor MPC-2. At *upper left* are three thick membranes continuous with endoplasmic reticulum (*arrows*). At *top center*, one of these thickened membranes is continuous with incomplete particle. This relationship between thickened membranes and incomplete particles was also observed in cells of plasma-cell tumor MPC-10. Lead subacetate stain. × 84,000

Electron micrograph by R. F. Zeigel, N.C.I.

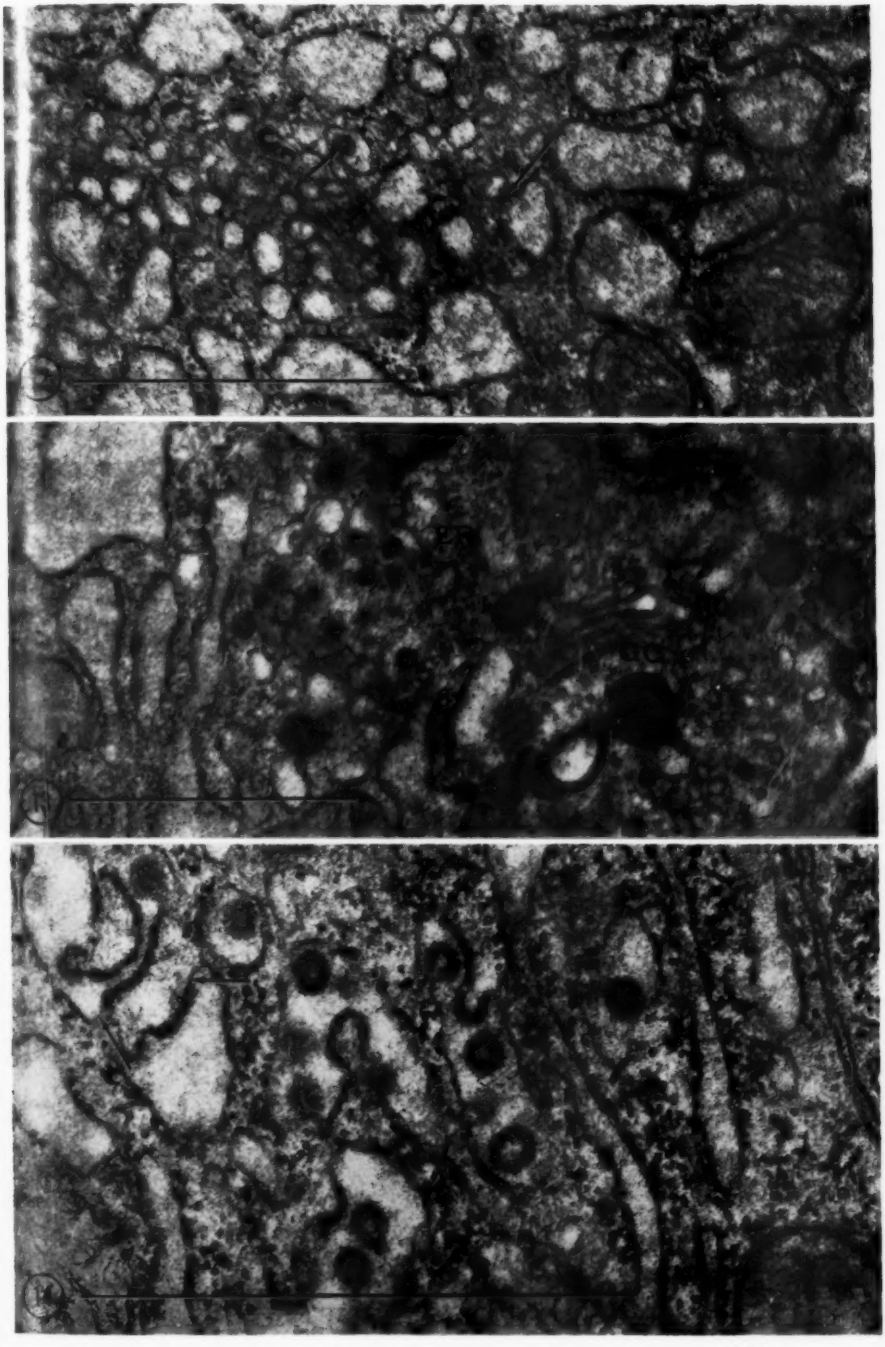


PLATE 179

FIGURE 15.—Part of cell of plasma-cell tumor X5563. Small part of another cell is at *upper right*. Golgi complex, occupying area from just below *center* of figure nearly to nuclear border at *lower left*, surrounds group of five bodies with ultrastructural characteristics of secretory granules. Above Golgi complex is area containing a number of type A-2 particles with relatively large centers, which appear to lie free in cytoplasm. A few particles of type A-1 seen in cells of other plasma-cell tumors are present in cisternae of ergastoplasm (*arrows*). Lead subacetate stain. × 35,000

FIGURE 16.—Area of juxtaposition of two cells of plasma-cell tumor X5563. Protruding under plasma membrane of lower cell are three particles, identifiable by their relatively large centers as the same as those in *upper part* of figure 15. Lead subacetate stain. × 53,000

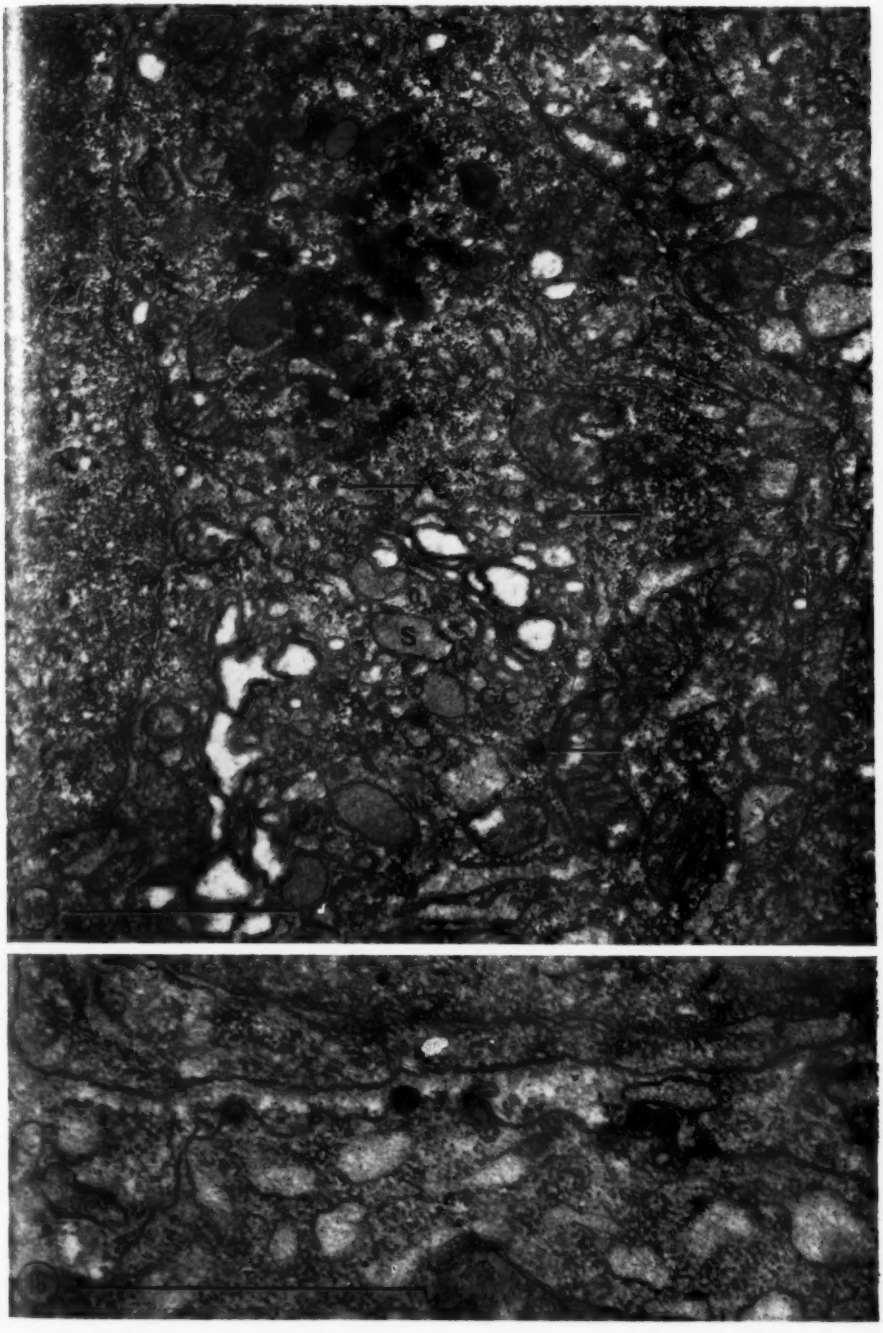

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PLATE 180

FIGURE 17.—Part of cytoplasm of cell of plasma-cell tumor X5563 containing numerous type A-2 particles. These particles differ from those characteristically observed in cells of plasma-cell tumors. They lie free in the cytoplasm, rather than within cisternae of ergastoplasm, and occasionally can be seen to be in continuity (*arrows*) with moderately electron-dense elongated bodies which sometimes possess a single and occasionally a double membrane. Particles have an average diameter of approximately 75 μ. *Note* granules in mitochondrion at *lower right*. Lead subacetate stain. $\times$ 90,000

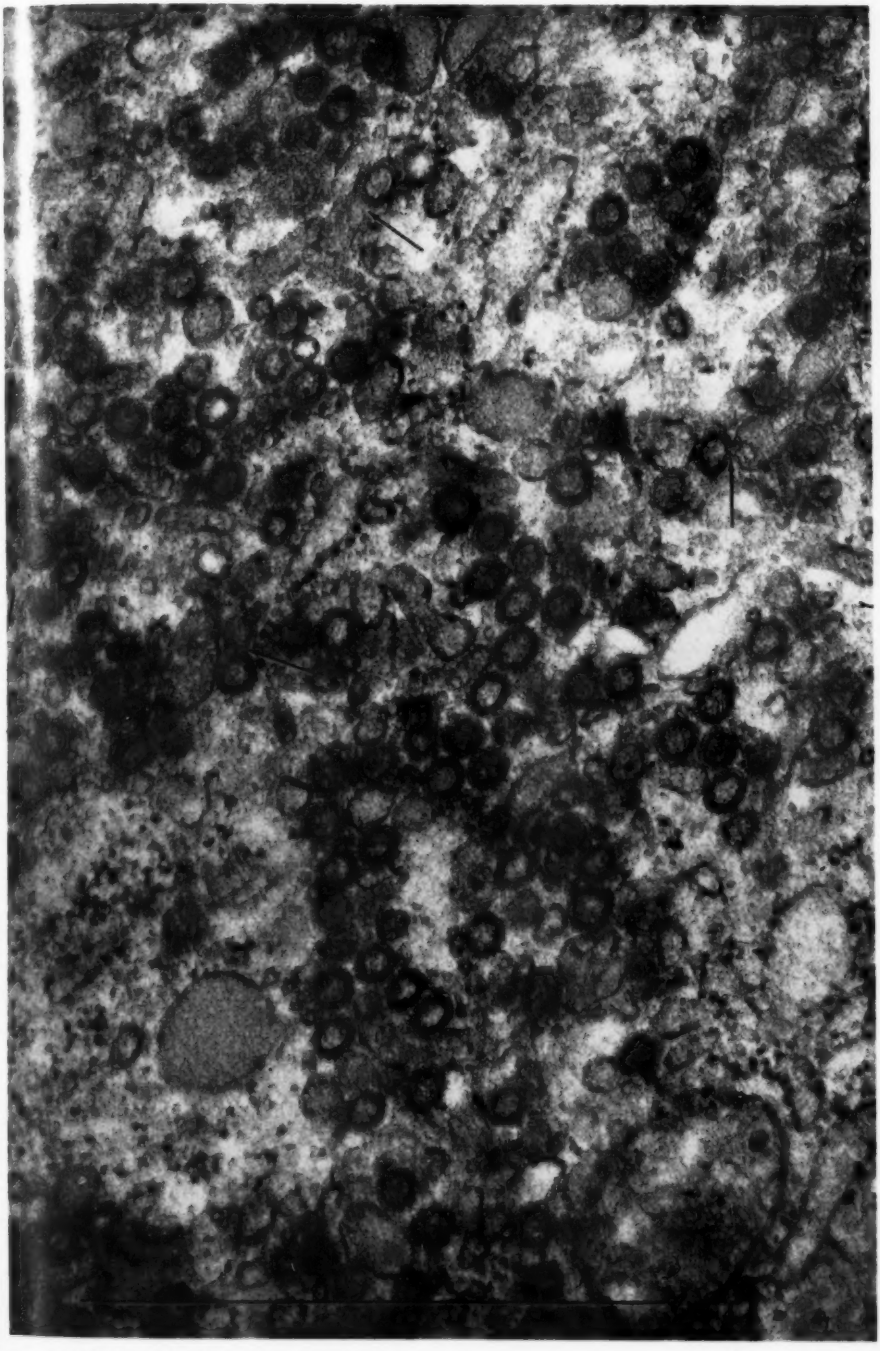
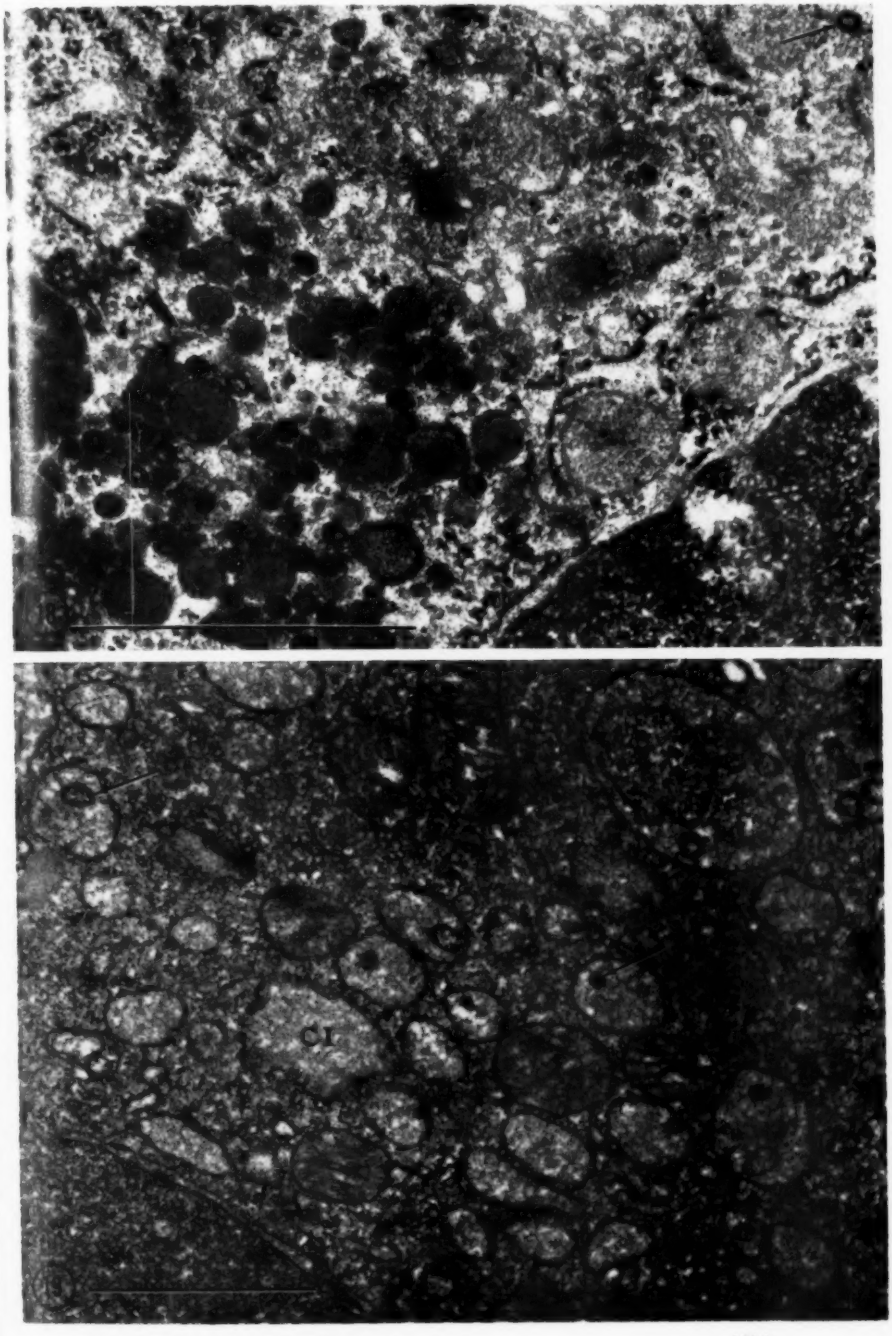


PLATE 181

FIGURE 18.—Part of cytoplasm of cell of plasma-cell tumor DPC-1. From *center* to *left* of figure is a group of particles having same location and measurements as those in figure 17 (type A-2). Bodies with ultrastructural features of secretory granules are also in this area. Type A-1 particle is in cisterna of ergastoplasm (*arrow*). Lead subacetate stain. × 53,000

FIGURE 19.—Part of cell of plasma-cell tumor DPC-1. Several of cisternae of ergastoplasm contain small circular bodies which vary somewhat in size (*arrows*). They are sections through cords or strands of ergastoplasm involuted into the cisternae. Lead subacetate stain. × 35,000



586399—61——20

PLATE 182

FIGURE 20.—Area of cytoplasm of cell of plasma-cell tumor Adj-PC-5. Golgi complex appears toward *upper left*. *Below* and to *right* are numerous bodies with ultrastructural features of secretory granules. Lead subacetate stain. × 53,000

FIGURE 21.—Part of cytoplasm of cell of plasma-cell tumor MPC-3′. To *left* of *center* a mitochondrion with its typical outer double membrane is continuous with single-membrane-bound body containing numerous small granules (*arrow*). Several other bodies of this type are present toward *right* of figure. Near *top* is cisterna of ergastoplasm, which contains many type A-1 particles. Lead subacetate stain. × 70,000

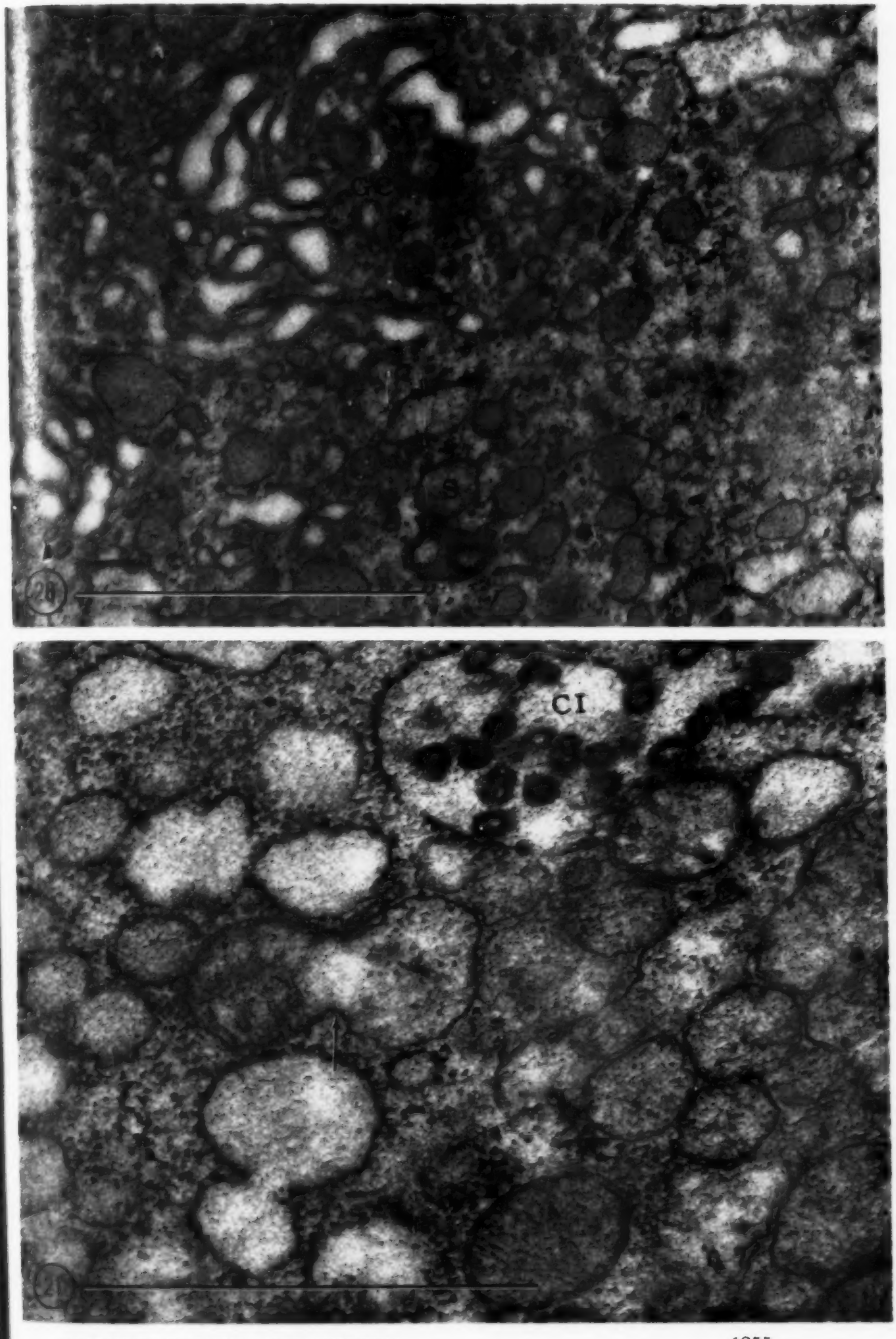

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PLATE 183

FIGURE 22.—Parts of several cells of plasma-cell tumor X5647. Portion of nucleus is at *right* and group of type A-1 particles is at *left*. Relatively few membranes of endoplasmic reticulum are present, the majority of ribosomes lying free in the cytoplasm. This feature was noted often enough in cells of this tumor to require comment. Lead subacetate stain. × 35,000

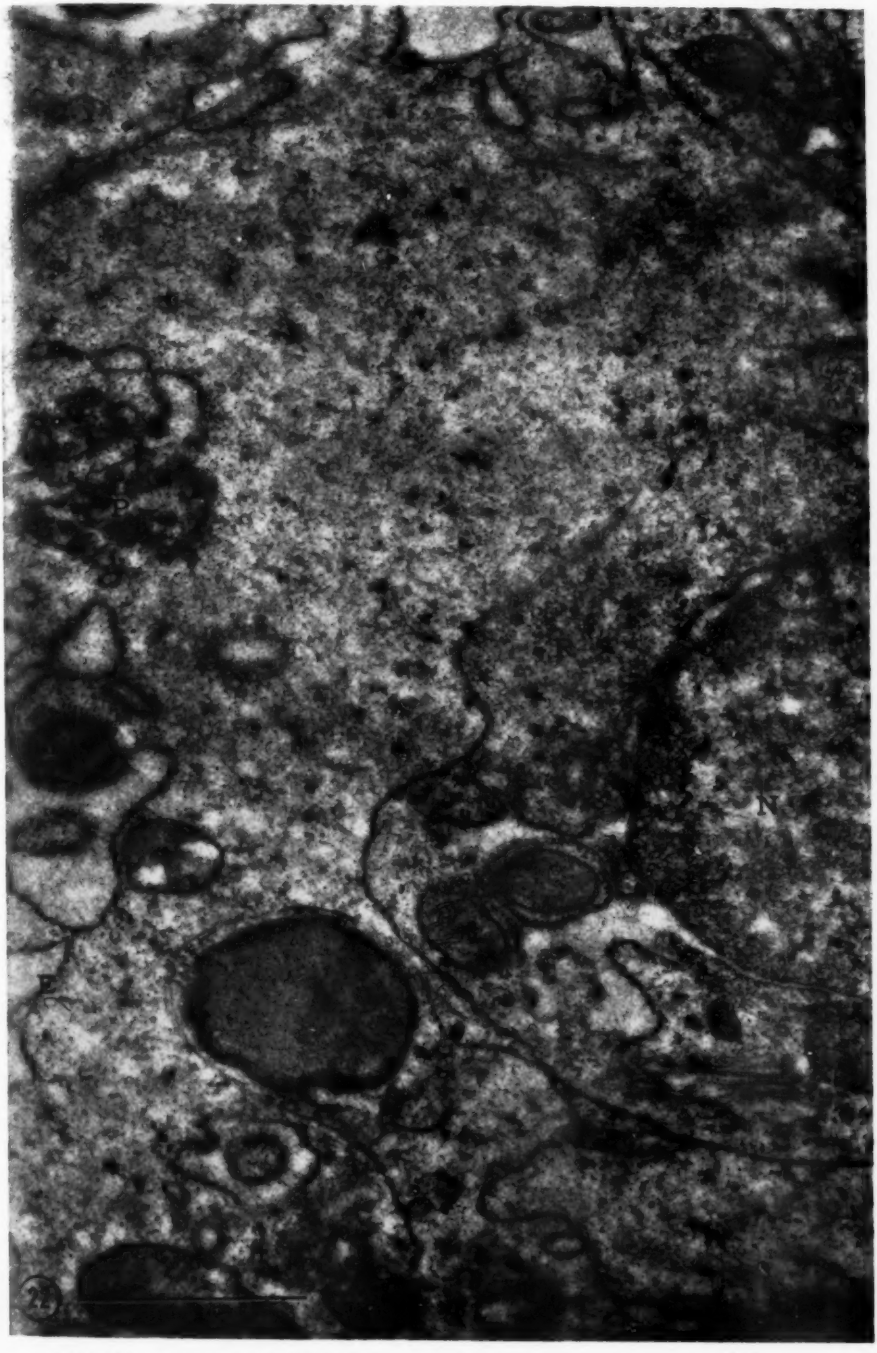
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PLATE 184

FIGURE 23.—Part of human multiple myeloma cell. Portion of nucleus appears at *bottom* of figure and immediately *above* a single centriole. Lead subacetate stain. × 35,000

FIGURE 24.—Part of human multiple myeloma cell. There are two centrioles *above* and to *right* of nucleus. Ergastoplasm typical of plasma cells is at *upper left*. Lead subacetate stain. × 26,000

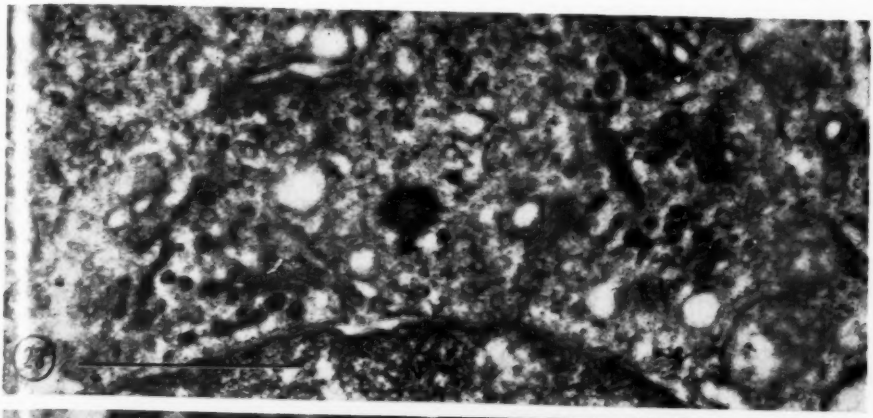
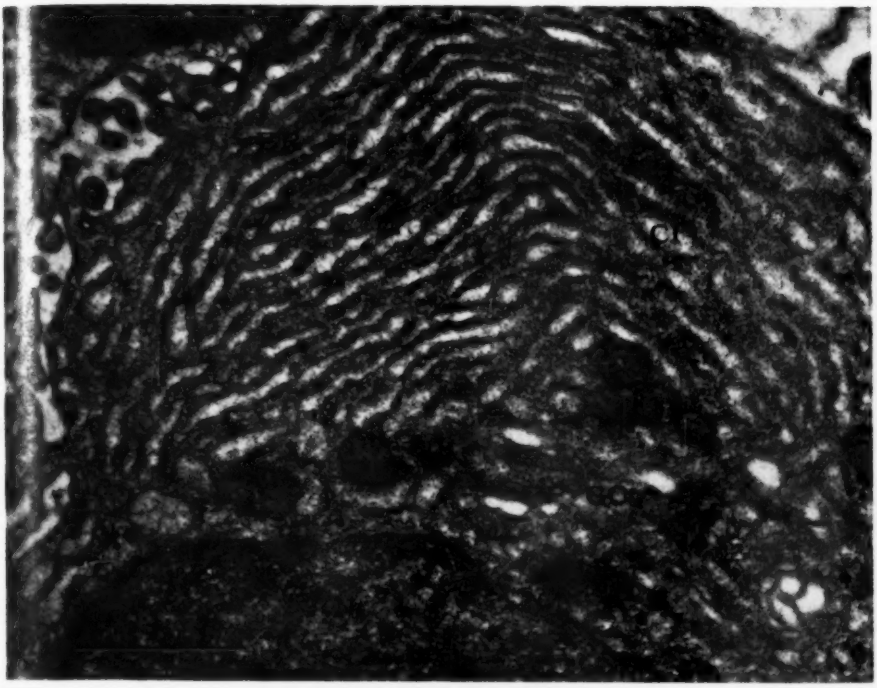


PLATE 185

FIGURE 25.—Part of human multiple myeloma cell. Portion of nucleus is at *bottom* of figure, and *above* and to *right* is part of Golgi complex. Most of figure is occupied by typical ergastoplasm with narrow cisternae. Several thin, tortuous cell processes are present at *upper left*. Majority of these cells examined exhibited similar processes. Lead subacetate stain. × 26,000

FIGURE 26.—Part of a cell of tumor 70429, originally diagnosed as undifferentiated plasma-cell tumor. As in this example, none of ultrastructural features characteristic of plasma cells were seen in any cells of this tumor examined. Only a few membranes of the endoplasmic reticulum are in evidence, large majority of the ribosomes lying free in cytoplasm. Lead subacetate stain. × 26,000



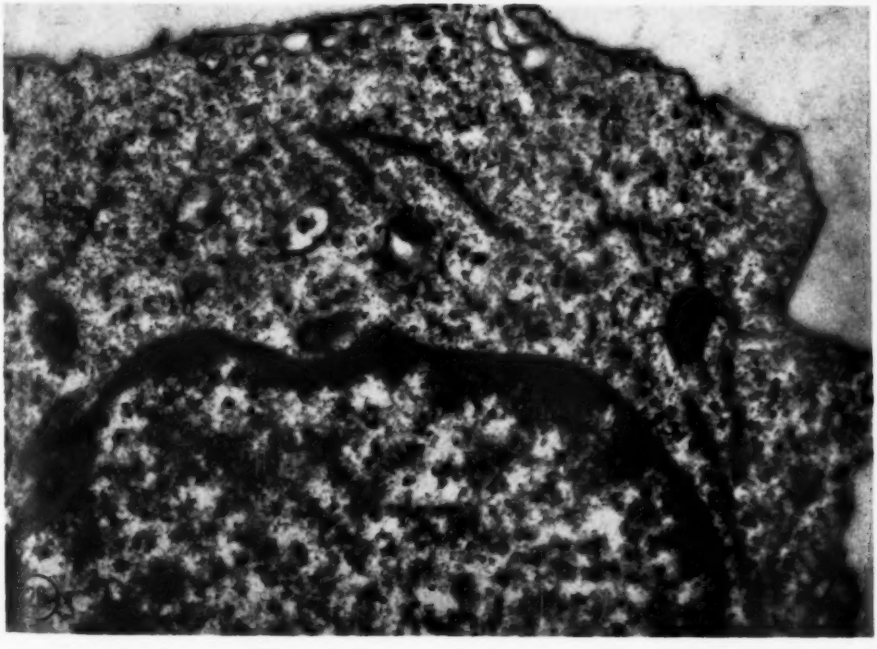


PLATE 186

FIGURE 27.—Region of nuclear "hof" in cell of tumor 70429. Profiles of flattened sacs and vesicles of Golgi complex lie on either side of two, possibly three centrioles. Lead subacetate stain. × 35,000

FIGURE 28.—Cytoplasmic area of a cell of tumor 70429 showing group of five unidentified bodies near *top center*. They do not possess the typical ultrastructural characteristic of virus particles but their membranes and internal substance have stained heavily. *Note* conspicuous small granules in mitochondria. Lead subacetate stain. × 53,000

FIGURE 29.—Parts of two cells of tumor 70429. Near *top* at point of contact between plasma membranes of two cells is large type "A" particle. It is rare to find these particles in an extracellular position. Lead subacetate stain. × 35,000

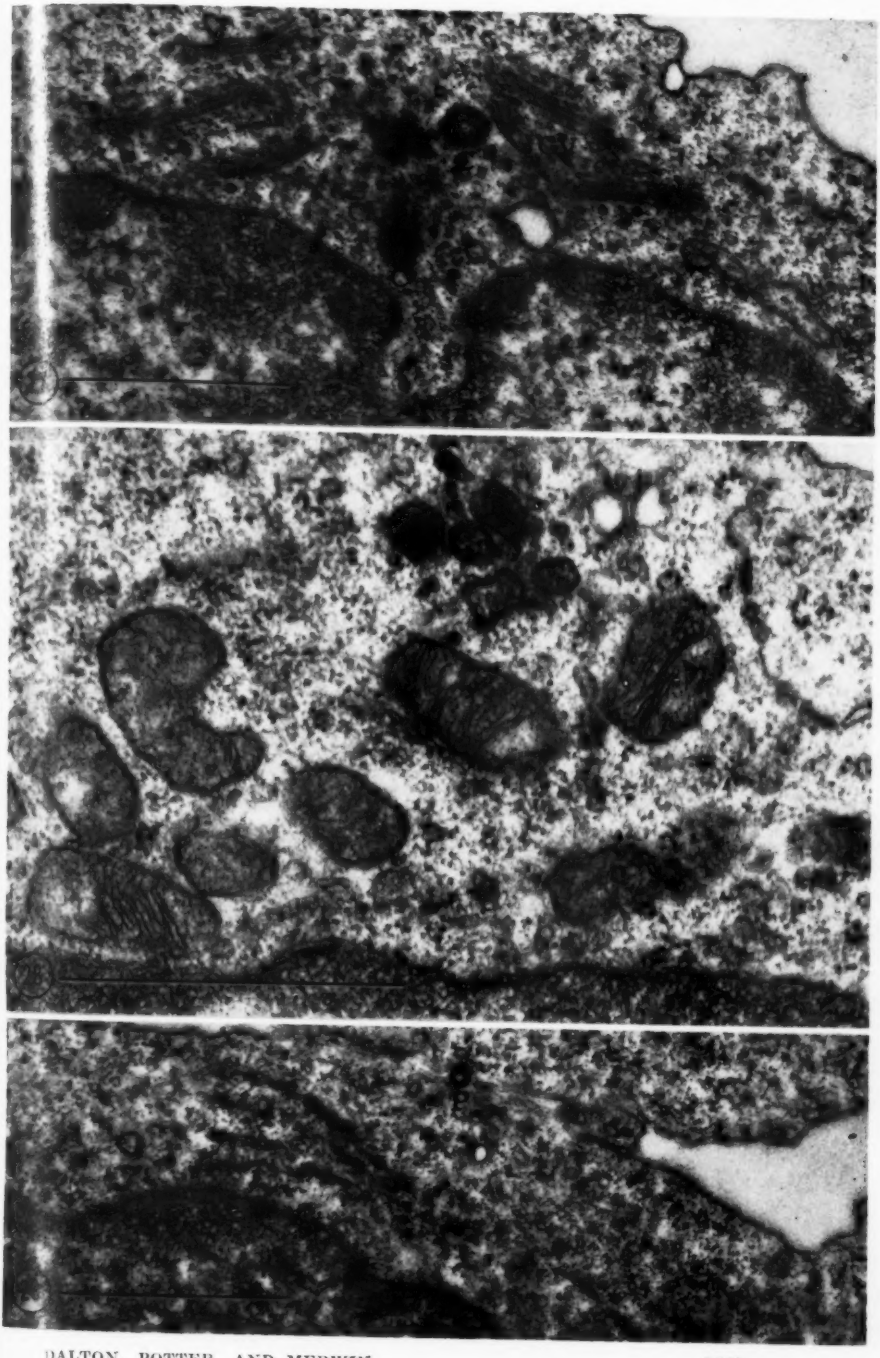
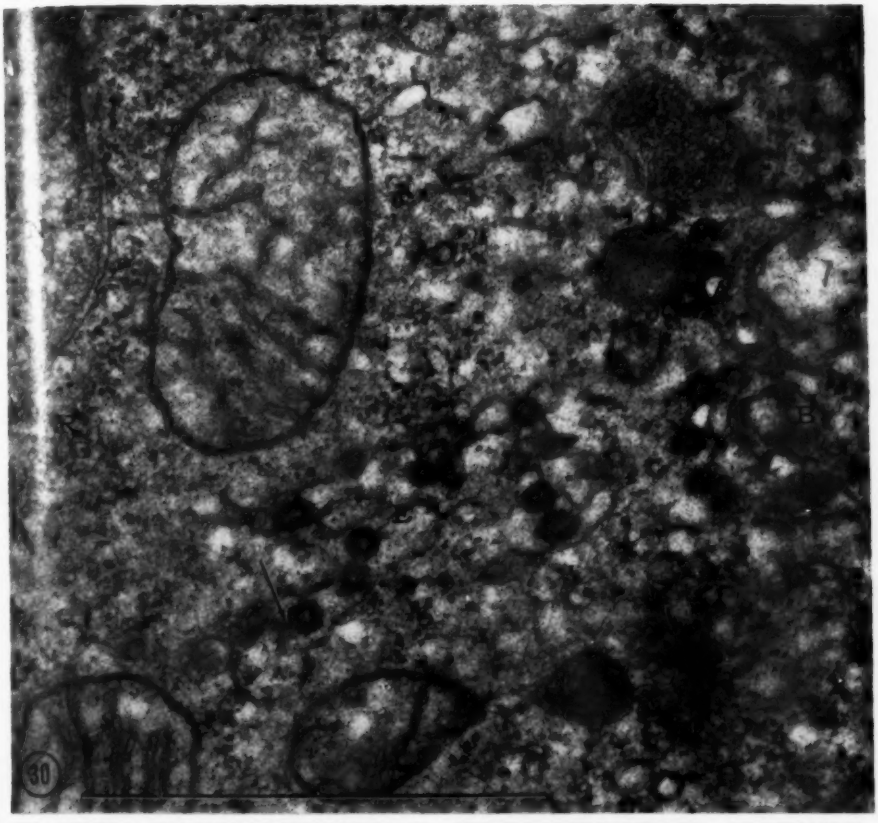


PLATE 187

FIGURE 30.—Part of cytoplasm and nucleus of cell of tumor H91061 which was diagnosed as undifferentiated plasma-cell tumor. The majority of ribosomes lie free in the cytoplasm. Only a few membranes of endoplasmic reticulum are present and those that are outline cisternae in which many type "A" particles are present. Some particles show continuity with the membranes (*arrow*). A doublet, *i.e.*, a particle with common outer membrane, but with two separate inner membranes, is at *A* and what appears to be a triplet in process of formation is at *B*. Individual particles average 78 mμ in diameter, which is somewhat greater than the type A-1 particles of cells of plasma-cell tumors. *Note* small granules in mitochondria at *upper left*. Lead subacetate stain. × 70,000

FIGURE 31.—Area of contact between two cells of tumor H91061 showing particles lying free in intercellular space. They have the general features of type A-1 particles, but their size is greater than that usually reached by this type, averaging 90 mμ in diameter. One particle contains dense nucleoid (*arrow*). Lead subacetate stain. × 70,000

FIGURE 32.—Point of apposition of two cells of tumor H91061 showing particles apparently in process of formation at plasma membrane. Lead subacetate stain. × 70,000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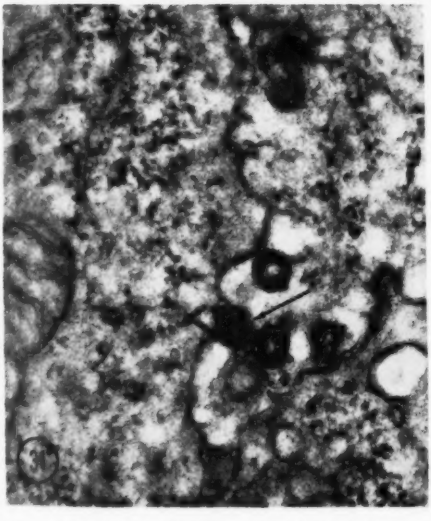
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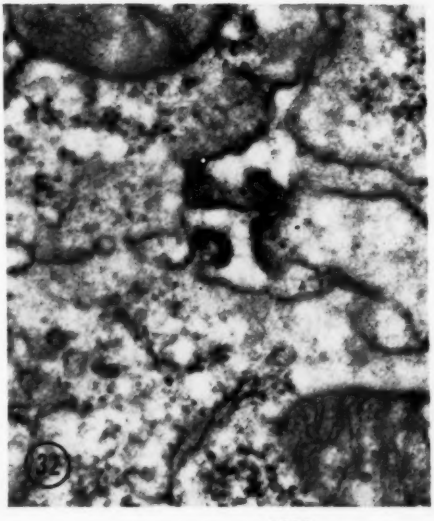
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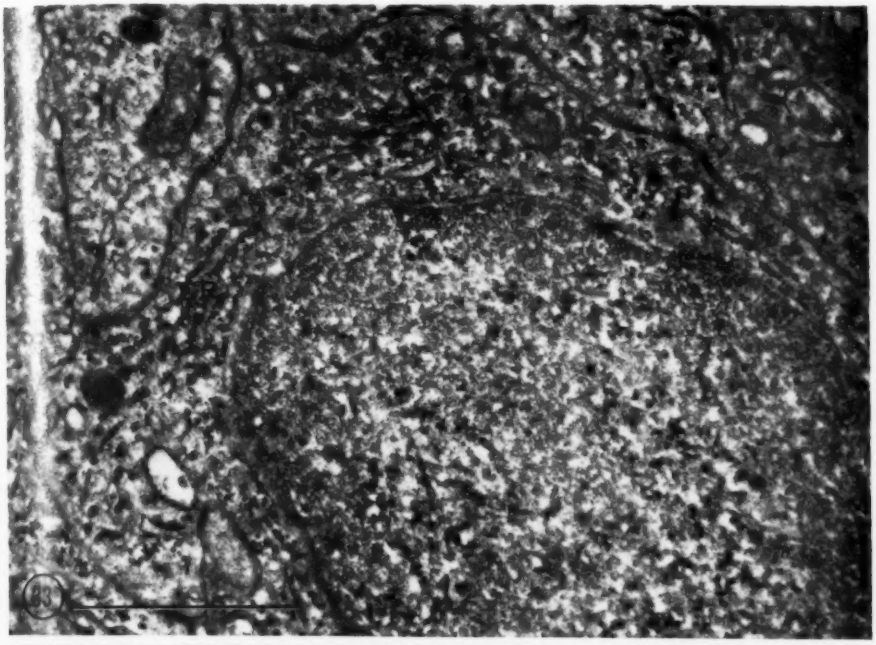
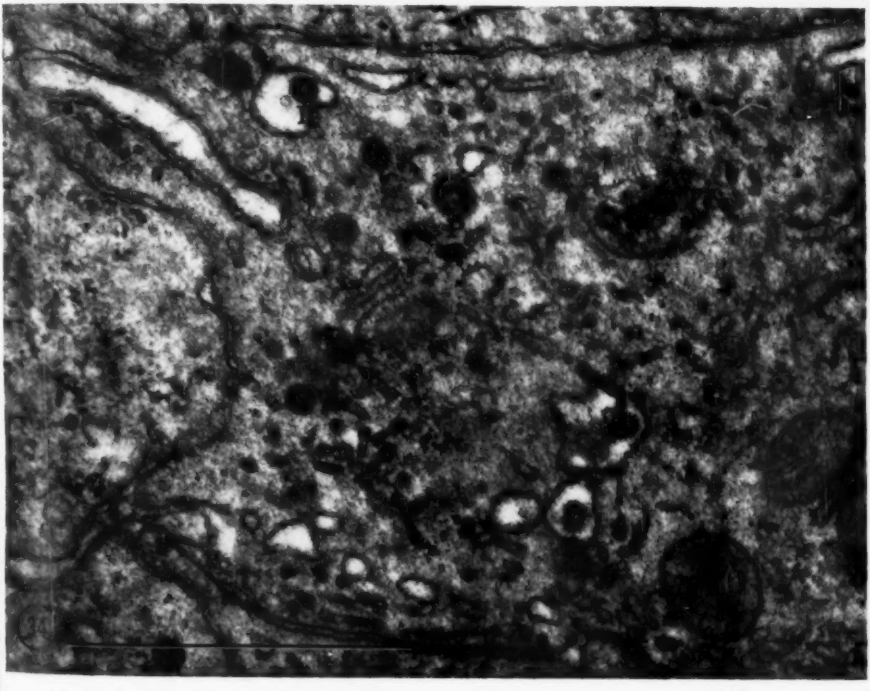
PLATE 188

FIGURE 33.—Parts of several cells of Sarcoma H93652 showing an amount of ergastoplasm greater than that characteristic of cells of tumor 70429. Lead subacetate stain. × 35,000

FIGURE 34.—Cytoplasm of cell of Sarcoma H93652 showing individual type "A" particles in small vacuoles. A group of three particles is in cytoplasm toward *right*. These particles are larger than those in cells of plasma-cell tumors since they average 90 mμ in diameter. Lead subacetate stain. × 53,000





"Hybrid" Type Cells in Combined Cultures of Two Different Mammalian Cell Strains [1]

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SUMMARY

Cells of a high-cancer N1 line derived from NCTC 2472 clone of mouse fibroblasts and cells of N2 or N2T' lines derived from low-cancer NCTC 2555 clone of the same origin were cultivated together *in vitro*. The evolution of mixed-cell populations was compared with the behavior of component cells in separate cultures. Separately cultivated, the cell lines kept their essential characteristics fairly stable. N1 cells maintained a karyotype with a modal number of nearly 55 telocentric chromosomes—one extra long. N2 lines had modal chromosome numbers of 62 and 58 chromosomes, 13 biarmed. Higher polyploids in pure cultures were recognized as doubles or quadruples of stemline cells. In mixed cultures the proportion of N1 cells progressively decreased until they almost completely disappeared. At the 3d month, a new type of cell designated "M" appeared, which later reached the proportion of one third to one fourth of the whole cell population. The M cells had many "hybrid" chromosomal and biological characteristics. They showed a modal number of 115 to 116 chromosomes with 9 to 15 metacentrics, frequently with one extra long telocentric. M cells from mixed populations and secondarily isolated M-type cell clones produced, in isologous mice, malignant tumors in which M cells could be identified. The possible origin of the "hybrid" type cells is discussed.—J. Nat. Cancer Inst. 26: 1269–1291, 1961.

SANFORD *et al.* (*1*) obtained, from a cell clone originating from normal subcutaneous tissue of the C3H mouse, several sublines or clones (*2*) having different morphological, biochemical (*3*), chromosomal (*4*), and tumor-producing characteristics.

We have recently (*5*) analyzed and compared cell lines derived from either the high-cancer NCTC 2472 clone (referred to as N1) or the NCTC 2555 clone (N2) having a particularly low cancer incidence. These two clones differed considerably in their static and dynamic cell morphology *in vitro*. They were also easily recognizable by the histological structure, growth pattern, and rapidity of tumors produced in the C3H mice.

[1] Received for publication December 22, 1960.
[2] We wish to thank Dr. Ephrussi (Paris) and Dr. Levan (Lund) for their stimulating interest and discussion in connection with this work, Dr. Gérard-Marchant for his help in the histological preparations, and Mr. Charlier for drawing the graphs.

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The N1 clone and its derived cell lines, all highly malignant, maintained constantly their pronounced fibroblast-like appearance, high mobility, and fan-shaped undulating membranes. The N1 karyotype, characterized by a modal number of nearly 55 telocentric chromosomes, appeared fairly stable during 20 months of culture *in vitro* in our laboratory. One of these chromosomes, constantly present, was very long (*5*). One metacentric, and rarely two, were present in no more than 26 percent of examined mitoses of the original N1 clone. The metacentrics disappeared nearly completely in the derived N1T line obtained after one passage of the N1 cells in the animal. Otherwise, cells recovered after from 1 to 7 passages were similar to the parental line. The N1 and derived lines had a marked tendency to produce higher polyploids which, however, maintained the typical, nearly pure telocentric chromosome pattern. These results compared fairly well with the previous chromosomal analysis of Chu, Sanford, and Earle (*4*).

Similarly, the N2 cells maintained their own features during 20 months of observation: rounded cells with regular undulating membranes or tentacular ramifications and a karyotype characterized by 54 to 67 chromosomes (modal number, 62), 9 to 19 of them biarmed (modal number, 13). These data (*5*) were on the whole similar to the results obtained with the same cells 2 years before by Chu, Sanford, and Earle (*4*), who observed, however, a somewhat higher modal number for this line: 69 chromosomes. Cells of two secondary lines, derived from the N2 clone after one animal passage, displayed some shift of chromosomal values, but remained unchanged for the essential feature of their metaphases: the modal number of 13 biarmed chromosomes. Morphologically these cell lines, despite some increase in their tumor-producing capacity, were very different from the N1 cell family, and the tumors they produced differed unequivocally from the N1 tumors in their histological aspect and slow, highly anaplastic growth.

Having accumulated data concerning the N1 and N2 cell families, especially regarding the extent of their normal variability *in vitro* and *in vivo*, we designed experiments to investigate the possibility of transfer of genetic material from one cell family to the other. To achieve this, we utilized prolonged culture *in vitro* of the 2 cell lines in close association. The behavior of combined cultures of N1 and N2 cells and the appearance in the mixed cultures of a new type of cells with "hybrid" chromosomal, morphological, and biological characteristics are presented. The results were reported in part in a preliminary note (*6*).

MATERIALS AND METHODS

Cultures of the high-cancer clone NCTC 2472 (N1) and the low-cancer clone NCTC 2555 (N2) were received from Dr. K. K. Sanford, Tissue Culture Section, Laboratory of Biology, National Cancer Institute, Bethesda, Maryland.

The newly designated N1T line, developed in our laboratory after one animal passage of the N1 cells, was in every respect similar to the parental line. The N2T′ line, obtained after one animal passage of the N2 clone and showing some increase of tumor-producing capacity, remained quite similar in its chromosome characteristics to the N2 cells (*5*). The cultivation, transfer, establishment of new cell lines *in vitro*, techniques of observation, recording of cell behavior *in vitro*, and the procedure used for chromosome studies were previously described in detail (*5*). Modified synthetic medium 199 (*7*), completed with 15 percent horse serum, was used uniformly for all cultures. Cells in mixed cultures were transferred exclusively by mechanical dispersion without trypsinization. Special precautions were taken to exclude any risk of cell contamination between cultures: Simultaneous handling of different cell cultures was avoided, and separate labeled supplies of media were prepared for each cell strain.

To check the tumor-producing capacity, we prepared standard 0.1 ml volumes of counted cell suspensions in the culture medium and inoculated them subcutaneously into 18- to 20-g males of the inbred C3H/He mice. Tumor growth was measured and recorded as previously described (*5*).

RESULTS

In preliminary experiments, mixtures containing 2.5×10^6 N2 cells and 1×10^6 or 1×10^5 N1 cells were inoculated subcutaneously into C3H mice. Rapidly growing tumors resulted. Histologically these tumors were all typical, spindle-cell sarcomas, undistinguishable from N1 tumors. Morphological and chromosomal studies of cell cultures obtained by explantation *in vitro* of these tumors confirmed that they were of N1 cell origin.

After the preliminary trials, mixed cultures, established with equal numbers of N1 and N2 cells, were grown *in vitro* for prolonged periods. In the experiment designated as "MS," 10^6 cells of the N1T line were associated with the same number of N2 clone cells. In another experiment designated "MT," 10^6 cells of the N1T line were associated with 10^6 cells of N2T′ line.

The mixed populations were maintained for several months in massive, actively multiplying cultures. We made observations directly by checking the appearance of the flask cultures and by qualitative and quantitative chromosomal analysis of secondary cultures prepared periodically on coverslips in flattened tubes designed in this laboratory (*8*). The data are represented in table 1.

If one assumes that the proportion of different mitotic types gives a satisfactory approximation concerning the different components of mixed culture, the results of these observations can be outlined as follows:

In both the MS and MT experiments, the N1 and N2 cells were present during the first 2 months of combined cultures. However, a selective advantage markedly favored the N2 cells that increased in proportion.

TABLE 1.—Evolution of cell components in mixed-cell population of MS and MT experiments

Experiment	Days of mixed cultures	Number of metaphases checked							
		Total	N1 type			N2 type			M type
			1 × s*	2 × s	4 × s	1 × s	2 × s	4 × s	
MS—mixed population established with N1T + N2 cells	55	57	10	12	2	29	4	0	0
	104	230	0	1	1	180	27	0	21
	139	54	0	4	1	38	3	2	6
	157	226	0	1	0	151	9	0	65
MT—mixed population established with N1T + N2T′ cells	122	75	15	13	2	32	13	0	0
	126	29	4	6	1	12	4	0	2
	147	97	12	22	1	15	23	1	23
	173	87	2	6	0	7	7	1	64
	204	115	0	6	0	23	2	0	84
	227	68	0	1	0	41	2	0	24

*s = "Stemline" hypotetraploid chromosome numbers: nearly 55 telocentrics for the N1 cells, nearly 60, with a modal number of 13 metacentrics for the N2 cells, and nearly 115, with 9 to 15 metacentrics for the M cells.

Likewise, the relative number of higher polyploids, especially of N1 type, increased. These could be easily recognized as double or quadruple stemline cells having multiples of normal chromosomal sets of either N1 or N2 complements. Thus, after 5 months of combined culture in the MS experiment, of the total 226 mitoses, only one N1 type could be found and it was a double stemline mitosis. After 7 to 8 months in the MT experiment, of 183 mitoses, only 7 higher polyploids of the N1 type were observed.

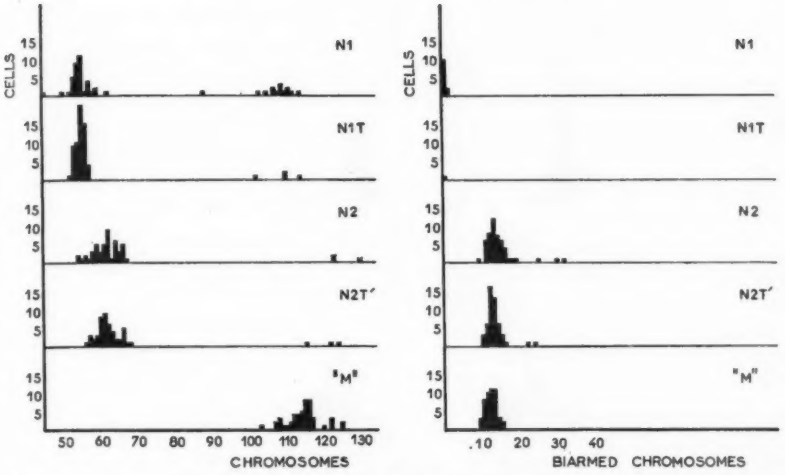
The evolution of the morphological aspect of the mixed cultures was consistent with these results. The characteristic elongated N1 cells progressively disappeared and were replaced by a population of rounded cells, some of them larger than the normal N2 cells and showing irregular, bizarre ramifications (figs. 1, 2, and 3). During the 3d month of the MS experiment and the 4th month of the MT experiment, a new cellular type appeared in the mixed cultures, which was designated the M cell. These cells, rare at the beginning, progressively increased in proportion. Their characteristics, according to detailed analysis of 48 M mitoses in the MT experiment, were as follows:

1) The chromosomes numbered between 103 and 125 but were grouped chiefly in the 115 or 116 area, which was very near the total of modal numbers of the N1- and N2-type cells (text-fig. 1*a* and figs. 4 through 10 and 12 through 14).

2) Biarmed chromosomes numbering 9 to 15 were present; their modal number, 12, coincides equally well with the values for the N2 cells, but was consistently below the corresponding values for the N2 polyploids (text-fig. 1*b*).

3) An extra long telocentric chromosome characteristic for the N1 cells was noted in 45 of the 48 checked M metaphases (figs. 8, 9, 10, and 14).

4) Once, at least, it was possible to identify singly the 11 biarmed chromosomes of an M cell with the 2 × 11 biarmed chromosomes of a



TEXT-FIGURE 1.—Frequency distribution of (*a*) chromosome numbers and (*b*) biarmed chromosome numbers of N1T, N2T', and M cells in the MT experiment; for comparison, data relative to the original N1 and N2 clones analyzed previously (*5*) are included. Each text-figure records data of nearly 50 analyzed mitoses.

polyploid N2 cell found in a mixed culture (figs. 6, 8, and 15). This particular analyzed M cell contained, as expected, the extra long chromosome, obviously absent in the polyploid N2 cell.

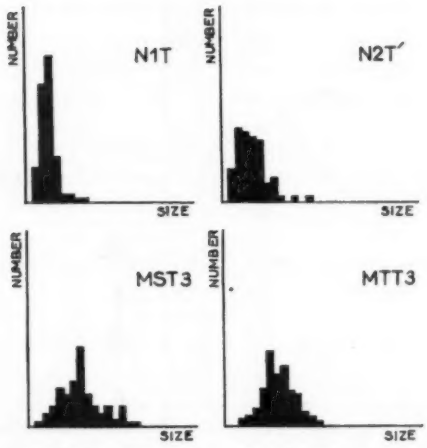
The M cells from the MS experiments had the same general characteristics as those from the MT series (figs. 7, and 8 through 10).

At the 8th month in the MT and the 5th month in the MS experiments, the mixed-cell populations were composed essentially of N2 and M cells. The latter reached a proportion of nearly one third of the whole population for the MT and one fourth for the MS experiment (table 1). At this stage, samples of the cells were inoculated subcutaneously in young C3H males. In both, rapidly growing tumors appeared at the inoculation site in high proportion of mice—6/7 for the MS and 12/12 for the MT series.

Curves representing the increase of mean diameter of these tumors approximated those obtained for the N1 tumors. On histological sections the tumors appeared as well-vascularized, round-cell sarcomas with some lobulation around wide vascular sinuses (figs. 18 and 19).

The M tumors were easily distinguishable from the typical spindle-cell sarcomas of the N1 type as well as from the anaplastic, round, and small cell tumors of the N2 type (figs. 16 and 17). Furthermore, the mean nuclear size of M tumor cells was, as shown in text-figure 2, significantly greater than that of either N1 or N2 tumors.

Five tumors of the MS and 2 of the MT experiments were explanted *in vitro* and the corresponding 7 established cell lines studied. Morphologically, the cells of these sublines were, with some minor differences, close to the final MS and MT cell population (figs. 3*a* and 3*b*). The chro-



TEXT-FIGURE 2.—Distribution of nuclear size in histological sections of tumors, produced with N1 and N2T′ cells and of two tumors obtained with MS and MT cell populations composed of N2 and M cells. Only M cells were found after explantation and chromosomal analysis of the MST3 and MTT3 tumors. Nuclear size is expressed in arbitrary values corresponding to planimetric measures. (*See* histological sections figs. 16 through 19.)

mosomal study of 6 of these lines always disclosed presence of cells having chromosomal numbers grouped chiefly in the 100 to 120 area (fig. 11). Of these chromosomes, 8 to 15 were biarmed. In approximately 40 percent of metaphases examined, the extra long telocentric chromosome could be identified. No correlation was found between the total number of chromosomes and the presence of the extra long telocentric chromosome. Generally, the M cells, after passage in animals, have shown a tendency toward wider dispersion and decrease of mean chromosome numbers. In 4 of the 6 analyzed lines, only the M-type cells were seen. In two others, some N1 cells were disclosed, but no persistence of N2-type cells could be found.

One of the MS tumor lines was cloned recently. Several clonal lines obtained from it are now under study. According to the results available at present, all the isolated clones have karyotypes with chromosomal numbers grouped chiefly in the 100 to 120 area, with modal numbers of 9 to 11 biarmed chromosomes. In at least one of these clones, the extra long telocentric is regularly present. All the 4 isolated M-cell-type clones so far assayed have produced tumors in 100 percent subcutaneously inoculated C3H/He mice with a standard dose of 2.5×10^6 cells per inoculum.

DISCUSSION AND CONCLUSIONS

Cultures of the NCTC 2472 (N1) and NCTC 2555 (N2) clones of mouse fibroblasts and their derived sublines were followed for many years in different laboratories, and the extent of their modifications in normal

conditions *in vitro* and *in vivo*, especially in respect to their karyotypes, was outlined (*4*, *5*).

From these data one can regard the appearance of the new peculiar M cell, present in mixed but not in separate, unmixed cultures of the N1 and N2 cells, as a result of close and prolonged association of these two cell types in actively mutiplying mass cultures *in vitro*. Similar results obtained independently in different experiments bear out this conclusion.

When we consider the properties of the M cell on the whole, its morphology and cancer-producing capacity, together with the "hybrid" character of its karyotype in which joined chromosome sets of the N1- and N2-cell types can be recognized, the hypothesis of a nuclear fusion with the production of "allopolyploid" cells has to be envisaged. In support of this hypothesis one can refer to the following data and considerations:

Lewis in 1927 (*9*) described and represented with precise drawings the formation of polynucleated cells by fusion of cytoplasm of individual mononucleated cells. Since then, this phenomenon, with a variety of animal cells, has been observed by other authors (*10–13*), and seems a frequent occurrence between cells having active ectoplasmic membranes (*14*), as is true with N1 and N2 cells. Furthermore, it is known that the mechanisms determining biosynthesis and nuclear division in polynucleated cells are to some extent synchronized. In these conditions, simultaneous formation of many mitotic spindles is not uncommon (*15*). As was observed and recorded by Lettré and Siebs (*16*) and Gey (*17*), 2 spindles can then fuse and the chromosomes of 2 nuclei rearrange themselves in one common equatorial plate. The mitosis can evolve further to form 2 or more nuclei containing "mixed" chromosomal sets.

These phenomena, when occurring under normal conditions in either wild heterogenetic or uniform homogenetic cell populations, will have no easily recognizable genetic and biological consequences. In reality, heteroploid cells issued from fusion of two different but genetically similar or undetermined cells would be hardly distinguishable from polyploids produced by endomitosis or C-mitosis. In the N1 and N2 cell association, many favorable conditions were available to facilitate the demonstration of this possible phenomenon. These conditions were biological and morphologic cell and chromosome characteristics related to these two kinds of cells, recognized in advance as very distinct and relatively stable.

Another essential factor was the apparent selective advantage of the M cell, first, over the N1 component in the mixed-cell population *in vitro* and, later, over the N2 component *in vivo*.

Thus, particular circumstances aided to recognize a phenomenon that probably can occur with a certain frequency in actively multiplying cell populations and that constitutes possibly one of the mechanisms of polyploidization of somatic cells. This mechanism was envisioned many years ago by Wilson (*18*), and recently, Schultz, discussing the malignancy of mammalian cells, again raised the possibility of nuclear fusion as one of the ways of genetic material transfer between somatic cells (*19*).

An alternative explanation of the origin of the M cell would be a pro-

found modification and rearrangement of the chromosomal complement of either N1 or N2 cells on an "autopolyploidization" basis. However, many considerations are against this interpretation. First, as already emphasized, the M-type cell was never observed in constantly checked, pure N1 or N2 cultures. One can hardly evisage the origin of M cells directly from the N1 cells with an abrupt formation of 10 or more metacentric chromosomes. The N2 cells, characterized as the M cells by the presence of many biarmed chromosomes, even when displaying in unmixed cultures a limited chromosomal shift and some increase of tumor-producing ability (*5*), never reached the high degree of malignancy proper to the M cells. Furthermore, the high polyploids in the pure N2 cultures have never shown a tendency to stabilize and to overgrow the N2 stem cells. This is consistent with the general trend manifested by high polyploids in pure, established cell lines to remain as a rather low and not increasing proportion of the whole cell population (*20*).

Another argument against the "autopolyploidization" hypothesis is that no transient forms between parental N1 or N2 cells and the M cell were ever seen in the primary mixed cultures. Such forms must be expected if one admits this hypothesis. According to studies by Levan and Biesele (*21*), Ford (*22*), Hsu and Klatt (*23*), and others, dealing with genetic evolution in cell populations *in vitro* controlled by constant karyotype analysis, chromosomal modifications in genetically homogeneous cell populations can usually be recognized by steps leading from simple polyploidization, through chromosomal rearrangements toward a stable, often aneuploid, karyotype. However, the opposite occurred with the M cells that appeared in an abrupt way and were closest to an ideal "hybrid" chromosomal complement from the beginning. Signs of rearrangement, such as loss of chromosomes and greater dispersion of their values, occurred later, especially after animal passages.

Another important point to be raised with regard to the nature of the M cell is its pronounced malignancy, expressed as a constantly high percentage of takes and rapidity of tumor growth. Therefore, the high cancer-producing capacity appears in the hybridization hypothesis as a dominant and stable character. This is especially apparent in the MS experiment in which the N2 component of the mixed culture was the original low-cancer NCTC 2555 clone.

This conclusion is supported by the recent results obtained with clones of M-type cells isolated after one animal passage of the MS mixed-cell populations. The features of these cells include high chromosome numbers grouped in the 100 to 115 area, with nearly 10 biarmed; their peculiar morphology and their invasiveness in the animal confirm the persistence of essential new properties acquired by the M cell.

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PLATES

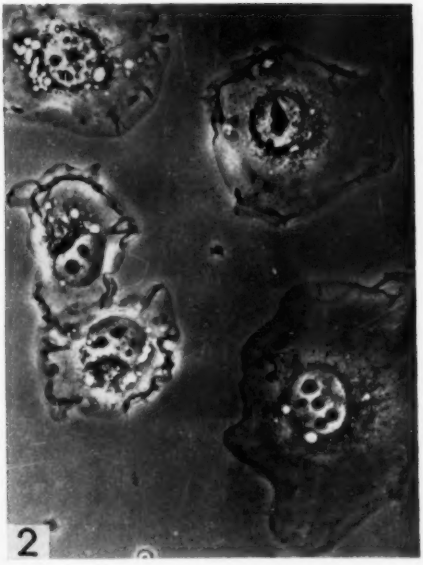
PLATE 189

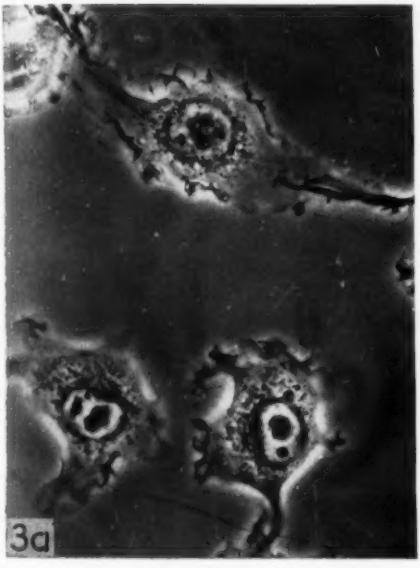
Phase-contrast microphotographs of living cultures. Approximately × 1,250

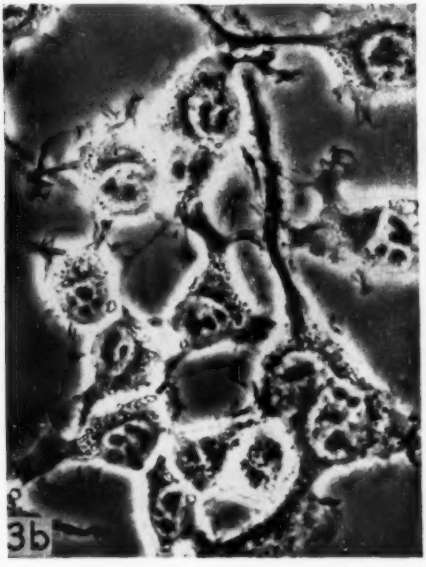
FIGURE 1.—Cells of high-cancer N1 line (NCTC clone 2472).

FIGURE 2.—Cells of low-cancer N2 line (NCTC clone 2555).

FIGURE 3 (*a* and *b*).—M-type cells (clone 2 and 1) derived from tumor (MST3) obtained in C3H mouse inoculated with MS mixed-cell population. All 279 mitoses checked after explantation of MST3 tumor *in vitro* were of M type.







586399—61——23

PLATE 190

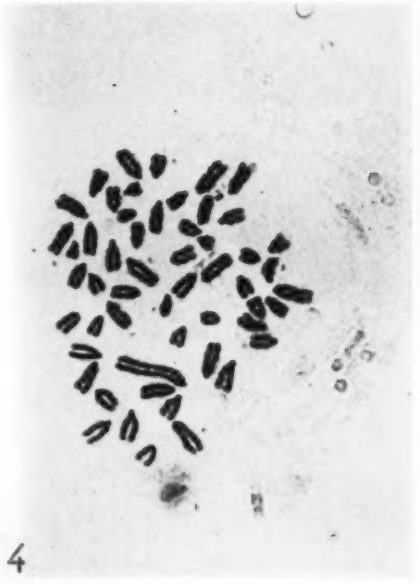
All figures are microphotographs of acetic-orcein-stained squash preparations. Approximately × 3,000

FIGURE 4.—Metaphase of typical N1T cell; 54 chromosomes, 1 extra long.

FIGURE 5.—Metaphase of N2 cell: 61 chromosomes, 12 biarmed.

FIGURE 6.—Polyploid N2 metaphase with recognizable double set of stem-cell chromosomes: 126 chromosomes, 22 biarmed.

FIGURE 7.—Metaphase of M-type cell from MS experiment: 113 chromosomes, 12 biarmed; 1 very elongated metacentric (*center*), 1 extra long telocentric (*arrow*).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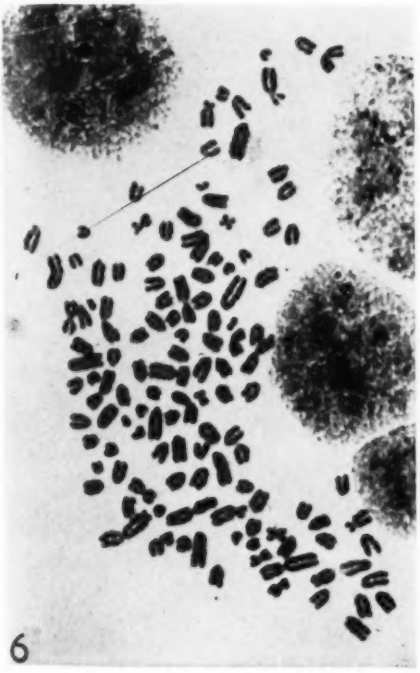
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PLATE 191

All figures are microphotographs of acetic-orcein-stained squash preparations. Approximately × 3,000

FIGURES 8, 9, and 10.—Metaphases of M-type cells from the MT experiment, containing, respectively, 113, 122, and 124 chromosomes—11, 14, and 13 biarmed. Extra long telocentric chromosome is present in each mitosis (*arrow*).

FIGURE 11.—Metaphase of M-type cell from MTT3 strain established *in vitro* after one animal passage of mixed-cell population from the MT experiment: 113 chromosomes, 10 biarmed, 1 extra long. All 127 mitoses checked in MTT3 strain cultures were of M type.

8

9

10

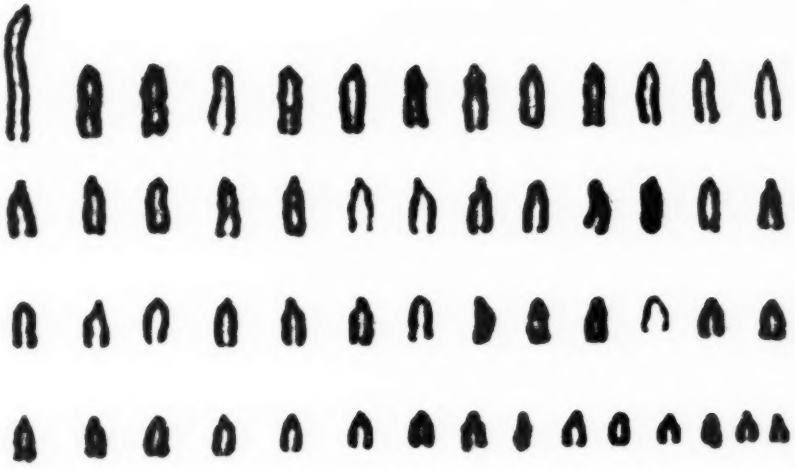
11

PLATE 192

Idiograms composed from microphotographs of acetic-orcein-stained preparations. Approximately × 5,500

FIGURE 12.—Idiogram of N1T metaphase (same as fig. 4): 54 chromosomes, all telocentrics, 1 extra long.

FIGURE 13.—Idiogram of N2 metaphase (same as fig. 5): 61 chromosomes, 12 biarmed.



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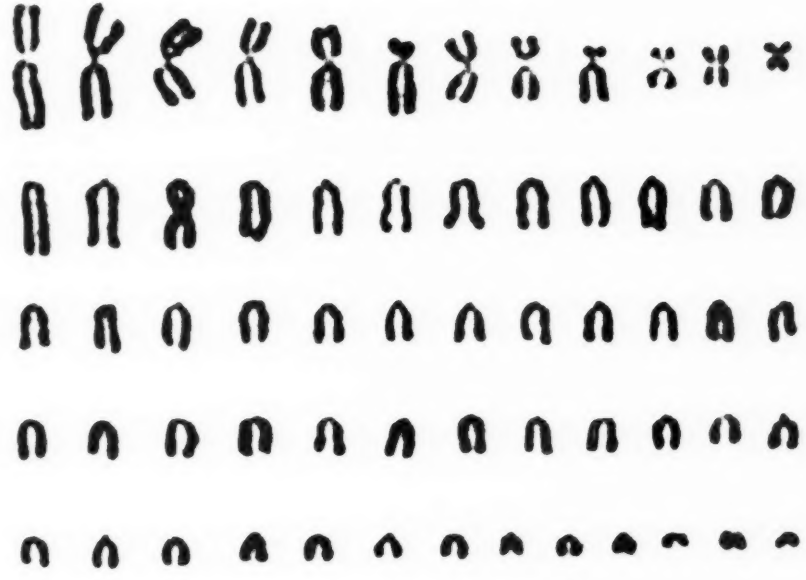

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PLATE 193

Idiogram composed from microphotograph of acetic-orcein-stained preparation. Approximately × 5,500

FIGURE 14.—Idiogram of M cell metaphase (same as fig. 8): 113 chromosomes, 11 biarmed, certain of them identifiable with metacentrics of the N2-type cell (fig. 13): 102 telocentric chromosomes, 1 extra long.

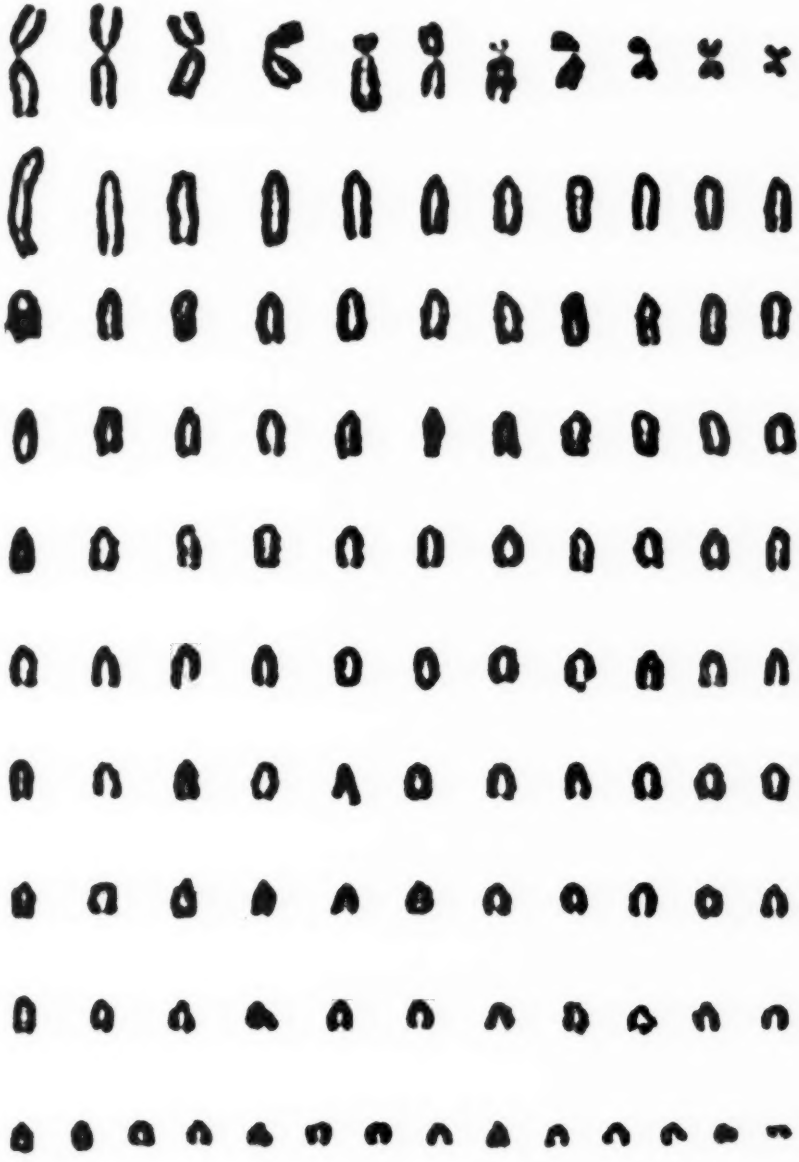

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PLATE 194

Idiograms composed from microphotographs of acetic-orcein-stained preparations. Approximately × 5,500

FIGURE 15.—Comparative idiogram of biarmed chromosomes from (*a*) M cell (same as fig. 8) and (*b*) polyploid (2 × *s*) N2 cell (same as fig. 6). Metacentrics of these two metaphases are fairly well identifiable.

a
b
15

PLATE 195

Histological sections of tumors obtained by inoculation of C3H males with 1 million of N1T (fig. 16), N2T′ (fig. 17), MS (fig. 18), and MT (fig. 19) cells. Alum hematoxylin-phloxine-saffron stain. × 470

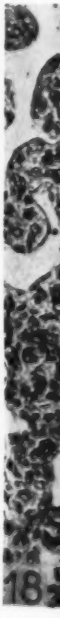
FIGURE 16.—Typical spindle-cell sarcoma characteristic of N1 tumors.

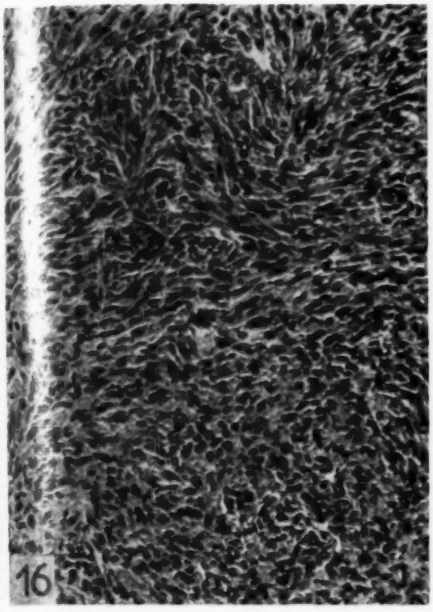
FIGURE 17.—Typical round-cell, anaplastic tumor of N2T′ type.

FIGURE 18.—One of tumors (MST3) obtained by inoculation of MS mixed-cell population.

FIGURE 19.—One of tumors (MTT3) obtained with MT mixed-cell population.

Nuclei of MST3 and MTT3 tumor cells are strikingly bigger than in N1 and N2T tumors. In cell lines established *in vitro* from these 2 tumors, only M-type mitoses were seen (279 and 127 mitoses checked).

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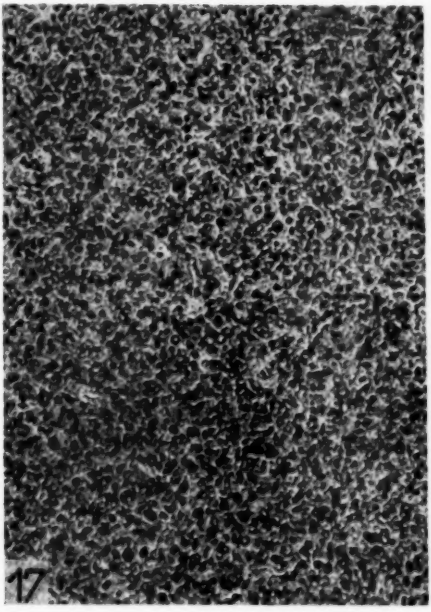
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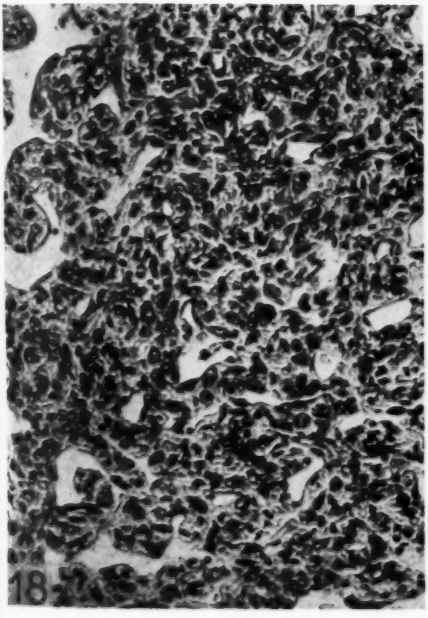
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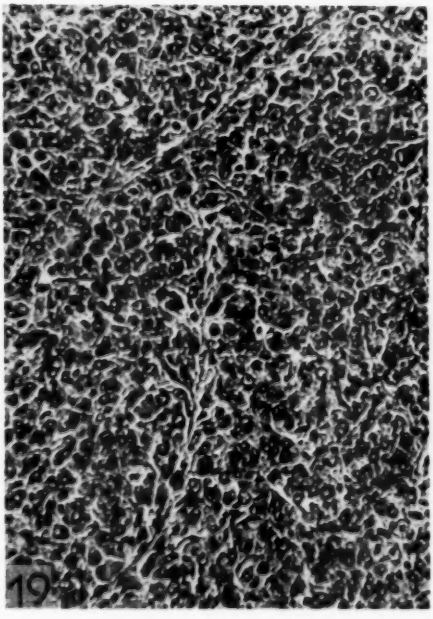
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Announcements

Gordon Research Conference

The Gordon Research Conference on Cancer will be held August 28 to September 1, 1961, at Colby Junior College, New London, New Hampshire. All applications for attendance must be submitted on the standard application form, with a deposit of $15. The Board of Trustees has established, for resident conferees, a fixed fee of $100 which covers registration, room, meals, and gratuities. Applications should be sent at least 2 months before the opening date. Requests for attendance or for additional information should be addressed to W. George Parks, Director, Department of Chemistry, University of Rhode Island, Kingston, Rhode Island. From June 12 to September 1 his address will be Colby Junior College, New London, New Hampshire.

NCI Monograph No. 5

National Cancer Institute Monograph No. 5, entitled "Carcinogenicity of *N,N'*-2,7-Fluorenylenebisacetamide," is scheduled for publication at the end of June, 1961.

The papers in the Monograph, all by authors from the National Cancer Institute, are: "Comparative Study of Cancer and Other Lesions of Rats Fed *N,N'*-2,7-Fluorenylenebisacetamide or *N*-2-Fluorenylacetamide," by Harold P. Morris *et al.;* "Atrophy, Proliferative Lesions, and Tumors of the Salivary Glands of Rats Ingesting *N*-2-Fluorenylacetamide or *N,N'*-2,7-Fluorenylenebisacetamide," by Katherine C. Snell *et al.;* "Intracranial Neurilemmoma and Medulloblastoma Induced in Rats by the Dietary Administration of *N,N'*-2,7-Fluorenylenebisacetamide," by Katherine C. Snell *et al.;* and "Carcinoma of the Glandular Stomach of Rats Ingesting *N,N'*-2,7-Fluorenylenebisacetamide," by H. L. Stewart *et al.*

Copies may be purchased from the Superintendent of Documents, Government Printing Office, Washington 25, D.C.

Manuscripts Approved for JNCI, March 15 to April 15, 1961

"Triadic labeling with I^{130}, I^{131}, and I^{133} for controlled determinations of tumor-localizing antibodies," by Eugene D. Day, Jakob A. Planinsek, and David Pressman.

"Clone strains of pig kidney cells with drug resistance and chromosomal markers," by Morgan Harris and Frank H. Ruddle.

"Treatment of total-body X-irradiated monkeys with autologous and homologous bone marrow," by B. G. Crouch, L. M. van Putten, D. W. van Bekkum, and M. J. de Vries.

"Pathologic changes in irradiated monkeys treated with bone marrow," by M. J. de Vries, B. G. Crouch, L. M. van Putten, and D. W. van Bekkum.

"Carcinogenic activity of analogues of *p*-dimethylaminoazobenzene. IV. Activity of the quinoline analogues," by Ellis V. Brown, Robert M. Novack, and Ali A. Hamdan.

"Effect of heparin on the circulating Walker 256 carcinosarcoma cells," by D. Agostino, C. E. Grossi, and E. E. Clifton.

"A study of host-virus relationship. I. Light, phase, and fluorescence microscopy of mouse-embryo cells infected with polyoma virus," by Elizabeth Bereczky, Leon Dmochowski, and Clifford E. Grey.

"Liver-cell proliferation in X-irradiated rats after single and repetitive partial hepatectomy," by G. F. Leong, R. L. Pessotti, and J. S. Krebs.

"Effect of hormones on the maintenance of hyperplastic alveolar nodules in the mammary glands of various strains of mice," by Satyabrata Nandi.

"Effect of hormones on mammary-tumor development from transplanted hyperplastic alveolar nodules in hypophysectomized-ovariectomized-adrenalectonized C3H/Crgl mice," by Satyabrata Nandi and Howard A Bern.

"Fatty acid composition of adipose tissue in patients with carcinoma of the breast," by William T. Dabney III.

"Intranuclear inclusions in tissue cultures infected with rat virus (RV)," by Clyde J. Dawe, Lawrence Kilham, and Willie D. Morgan.

U. S. GOVERNMENT PRINTING OFFICE: 1961